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TITLE: Transcription Activator Reprogramming Gene Therapy (TARGET) of Breast Cancer Cells with Adenoviral Vectors for Interferon Regulatory Factors

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14. ABSTRACT Transcription factor reprogramming gene therapy of cancer results in upregulation of numerous genes which result in expression of numerous antitumor cytokines and chemokines, as well as a host of proapoptotic proteins and other proteins that mediate cell death or terminal differentiation. This allows for the application of a single vector to achieve expression of a multitude of genes that could only be achieved by application of an impossibly large number of separate vectors. We have termed this concept as Transcription Activator Reprogramming GENE Therapy, or TARGET. We and others have shown that certain Interferon Regulatory Factors upregulate numerous antitumor genes in cancer cells, which results in expression of numerous cytokines, chemokines, and proapoptotic factors that result in a marked antitumor response, both in terms of cell death and enhanced immune response against the cancer. We explored the concept that adenoviral vectors for IRF-3 can reprogram breast cancer cells to kill themselves, resulting in eradication of the treated breast cancer cells by apoptosis.					
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INTRODUCTION:

Unlike standard gene therapy where one gene is upregulated by administration of a single vector, nuclear transcription factor reprogramming gene therapy of cancer results in upregulation of numerous genes which result in expression of numerous antitumor cytokines and chemokines, as well as a host of proapoptotic proteins and other proteins that mediate cell death or terminal differentiation. This allows for the application of a single vector to achieve expression of a multitude of genes that could only be achieved by application of an impossibly large number of separate vectors. We have termed this concept as Transcription Activator Reprogramming Gene Therapy, or TARGET treatment of cancer. We and others have shown that certain Interferon Regulatory Factors upregulate numerous antitumor genes in cancer cells, which results in expression of numerous cytokines, chemokines, and proapoptotic factors that result in a marked antitumor response, both in terms of cell death and enhanced immune response against the cancer. While performing microarray analysis of our adenoviral vector for Interferon Regulatory Factor-1 in breast cancer cells, which we have published to result in marked apoptosis of breast cancer cells, we serendipitously found more impressive massive upregulation of numerous cytokines and chemokines which may enhance the immune response against breast cancer. In other published studies, Interferon Regulatory Factor-3 has been found in stable transfection experiments in melanoma to result in upregulation of numerous cytokines and chemokines which results in an antitumor immune infiltrate. We explored the concept that adenoviral vectors for IRF-1 and IRF-3 can reprogram breast cancer cells to kill themselves and to enhance the host immune recognition against themselves, resulting in not only the eradication of the treated breast cancer cells, but also eradication of metastases and prevention of recurrence.

BODY:

TASK 1 involved ex vivo infection of C3L5 mouse breast cancer cells and injection into syngeneic C3H/HeJ mice with tumor assessment and then rechallenge with uninfected C3L5 cancer cells (months 1-3). Due to problems with the availability and characterization of Ad-IRF-3, months 1-3 were involved in performing these studies before this TASK.

Propagation and characterization of Ad-IRF-3(WT), Ad-IRF-3(5D), and Ad-IRF-3(DN), and functional expression of IRF-3(WT) and IRF-3(5D)

To fully assess the capacity of IRF-3 to limit breast cancer cell growth, we obtained recombinant adenovirus (rAd) encoding wild-type human IRF-3 (Ad-IRF-3(WT)) which is the native protein that requires activation by phosphorylation, and rAd encoding a constitutively active IRF-3 in which residues at positions 396, 398, 402, 404, and 405 are replaced by the phosphomimetic aspartate amino acid (Ad-IRF-3(5D)), which results in constitutive translocation of IRF-3 and transcriptional activation in the nucleus (Lin et al., 1999). Inactive wild-type IRF-3 constitutively shuttles in out of the nucleus, whereas phosphorylation within the serine/threonine cluster (and replacement with the phosphomimetic aspartate) induces a conformational change in IRF-3 that allows homo- and hetero-oligomerization, nuclear localization and association with the coactivator CBP/p300 which retains IRF-3 in the nucleus and induces transcription of IFN α/β genes and other IFN-stimulated genes (Lin et al., 1999). Finally, we also obtained rAd encoding a truncated IRF-3 which acts as a dominant negative mutant (Ad-IRF-3(DN)). These rAds were a generous gift from M. Riviaccio (Riviaccio et al., 2005) and propagated and then characterized below.

We previously confirmed high efficiency of adenoviral gene transfer in breast cell lines using a recombinant adenovirus expressing the enhanced green fluorescent protein (Ad-EGFP), which is readily identified under fluorescent microscopy and flow cytometry. Cells infected with Ad-EGFP displayed greater than 95% infectivity at a multiplicity of infection (MOI) of 100 in C3-L5 mouse breast cancer cells and MOI of 25 in MDA468 human breast cancer cells (Kim et al., 1994; Pizzoferrato et al., 1994).

In order to assess the production of human IRF-3 and not endogenous IRF-3 in cells transduced with the rAds, we transduced mouse breast cancer cells with the rAd vectors that produce human IRF-3 products and immunoblotted using an antibody against human IRF-3 that does not cross react with mouse IRF-3. Immunoblotting demonstrated that no infection (NI) and Ad-Null infection produced no detectable human IRF-3 protein in C3-L5 cells, while Ad-IRF-3(WT) and Ad-IRF-3(5D) produced clearly detectable IRF-3 product (Figure 1). Ad-IRF-3(DN) produced a smaller molecular weight immunodetectable product consistent with the truncated non-functional dominant negative IRF-3, and Ad-IRF-1 does not generate significant IRF-3 (Figure 1).

IRF-3 is most well known for transcriptional upregulation of Type I IFN expression (Parekh and Maniatis, 1999; Civas et al., 2006). To confirm that IRF-3 protein expressed in cells was functional, we measured expression of IFN- α and IFN- β by the human IRF-3 transduced by the rAds in the mouse breast cancer cell line C3-L5, as well as the human breast cancer cell line MDA468. As seen in Table 1, Ad-IRF-3(WT) infection resulted in markedly increased levels of mouse IFN- α and IFN- β in the mouse C3-L5 cell line, confirming that IRF-3(WT) can be functional, although this was somewhat surprising given that IRF-3(WT) would normally require phosphorylation to translocate to the nucleus and upregulate IFN response products such as IFN- α and IFN- β . Interestingly, Ad-IRF-3(5D) only appeared to increase IFN- β in the mouse breast cancer cell line, with no significant increase in IFN- α .

As seen in Table 2, Ad-IRF-3(WT) in MDA468 breast cancer cells, a human cell line, did not induce either IFN- α or IFN- β despite high expression of IRF-3 in MDA468. This would be consistent with the need for activation by phosphorylation for transcriptional activity. Ad-IRF-3(5D) once again demonstrated marked IFN- β production as measured by ELISA, once again confirming the constitutive functional expression by Ad-IRF-3(5D). Ad-IRF-1 is known to increase IFN- α production in both C3-L5 (Kim et al., 2004) and MDA468 (Pizzoferrato et al., 2004), and acted as a positive control. There were relatively insignificant IFN expression above baseline with no infection (NI), empty vector control (Ad-Null), and dominant negative (Ad-IRF-3(DN)) infected cells.

IRF-3(5D) induces apoptosis in breast cancer cells

Programmed cell death, or apoptosis, can be induced by multiple cell signaling mechanisms (Green and Evan, 2002), and IRF-3 has been previously reported to induce apoptosis in other cell types (Heylbroeck et al., 2000; Romieu-Mourez et al., 2006), but not in breast cancer cells. The high efficiency of IRF-3 gene transfer by Ad-IRF-3(WT) and Ad-IRF-3(5D) allowed us to examine the entire population of breast cancer cells for apoptosis. Annexin V binding to cell-surface phosphatidyl serine is one hallmark of apoptotic cell death (Koopman et al., 1994), and propidium iodide (PI), which stains DNA, is a marker for permeabilized, necrotic cells. Compared to no or Ad-Null infection, MDA468 cells displayed a profound increase in annexin V-positive as well as PI-positive cells after infection with Ad-IRF-3(5D) (Figure 2). Ad-IRF-3(WT) appears to induce some level of apoptosis since the Annexin V

positive fraction(both early and late apoptosis) totaled 19%, but this pales in comparison to the 60% total seen with Ad-IRF-3(5D), and the 69% total seen with Ad-IRF-1, our positive control (Pizzoferrato et al., 2004).

To further confirm that IRF-3 expression resulted in cell death by an apoptotic mechanism via caspases, we assayed for caspase-3 and caspase-7 cleavage by immunoblotting. As seen in Figure 3, NI, Ad-Null, and Ad-IRF(DN) do not cause any evidence of caspase cleavage of caspase-3 or caspase-7, the primary effector caspases seen in apoptosis. Ad-IRF-3(WT) does cause some detectable caspase-3 and possibly caspase-7 cleavage, but Ad-IRF-3(5D) causes significantly more caspase-3 and caspase-7 cleavage on par with Ad-IRF-1 for caspase-3 cleavage, and clearly detectable caspase-7 cleavage.

Ex vivo infection of C3L5 with Ad-IRF-3(5D) then tumor cell implantation in syngeneic mice.

We then performed an abbreviated TASK 1 focusing on Ad-IRF-3(5D). Mice were injected with C3L5 cells infected ex vivo with either Ad-IRF-3(5D) (moi 200), and Ad-Psi5 (empty vector, moi 200) or mock infection (no virus) at 5×10^5 cells per animal. 6/6 animals injected with C3L5 infected with Ad-Psi5 developed tumors while only 1/6 animals injected with C3L5 infected with Ad-IRF-3(5D) developed tumors at 60 days. We then challenged all animals with uninfected C3L5 at 5×10^5 cells per animals, and all animals developed tumor which resulted in the termination of this abbreviated TASK.

TASK 2 involved intratumoral injection of highest recombinant adenoviral doses possible (months 4-6). Mice were injected with 5×10^5 C3L5 cells and tumors followed. Mice were randomized to equal average tumor size and then injected intratumorally with the highest concentration of adenovirus possible in 100 microliter and tumors followed. Tumors were to be reinjected if necessary. As seen in Figure 4, Ad-IRF-1 and Ad-IRF-3 were equally effective in slowing the growth of established tumors in the C3L5 mouse model when injected three times. Interestingly, the combination of Ad-IRF-1 and Ad-IRF-3 actually seemed to worsen the antitumor effect (data not shown).

IRF-3(5D) expression suppresses growth of mammary tumors in vivo

Because we even found an antagonistic effect of Ad-IRF-1 and Ad-IRF-3 in vivo in our syngeneic model, and because we actually had three versions of Ad-IRF-3 to compare, and finally because we had identified apoptosis as a key antitumor event with Ad-IRF-3, we decided to modify TASK 2 to a xenogeneic SCID-Bg model. Subcutaneous intra-tumoral injections of rAd (2×10^8 pfu) every other day for three injections were administered to female SCID-Bg mice with established MDA468 tumors in the mammary line. SCID-Bg mice lack B cells, T cells, and NK cells, and therefore provide an optimal model for a xenogeneic model for a human breast cancer cell line in mice, and helps focus the study of apoptosis in vivo. Ad-IRF-3(5D) caused marked tumor suppression of MDA468 tumor growth in SCID-Bg mice, while Ad-IRF-3(WT) appeared to cause an intermediate tumor suppression, compared to Ad-Null injected and Ad-IRF-3(DN) injected animals (Figure 5).

At this point we had extended the research greater than one year and had depleted the funds provided for further work on this project.

KEY RESEARCH ACCOMPLISHMENTS:

- Propagation of multiple forms of Ad-IRF-3
- Characterization of multiple forms of Ad-IRF-3
- Establishment of the apoptotic effect of Ad-IRF-3 in breast cancer cells
- Mechanistic analysis of the apoptotic effect of Ad-IRF-3 in breast cancer cells
- Establishment of the antitumor effect of different forms of Ad-IRF-3 in both syngeneic and xenogeneic models of breast cancer

REPORTABLE OUTCOMES:

1) Manuscript-

- a. (in preparation) "RECOMBINANT ADENOVIRUS EXPRESSING CONSTITUTIVELY ACTIVATED INTERFERON REGULATORY FACTOR 3 CAUSES APOPTOSIS AND TUMOR GROWTH SUPPRESSION IN BREAST CANCER MOUSE MODELS"

2) Abstracts/presentations-

- a. Sung K-Y, Stang MT, Ren B, Armstrong MJ, Liu Y, **Yim JH**. IRF-3, a mediator of innate immunity, causes tumor suppression of cancer in vitro and in vivo. Academic Surgical Congress, Phoenix, AZ, February 2007.
- b. Stang MT, Sung K-Y, Ren B, Armstrong MJ, Liu Y, **Yim JH (presenter)**. Recombinant adenovirus expressing constitutively activated IRF3 suppresses tumor growth in a human breast cancer model. Central Surgical Association, Chicago, IL, March, 2007

CONCLUSION:

We have demonstrated that Ad-IRF-3 in the constitutively active form (5D) is the most potent rAd for inducing apoptosis in vitro in breast cancer cells. It appears to have equivalent antitumor activity in vivo as Ad-IRF-1, but there is no additive or synergistic effect between the two. Further characterization of the apoptotic effect and possibly the immune effect of Ad-IRF-3 may allow us to find ways to improve the antitumor effect.

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Rivieccio MA, John GR, Song X, Suh HS, Zhao Y, Lee SC, Brosnan CF. (2005) *J Immunol.* **174**(6):3719-26
Romieu-Mourez R, Solis M, Nardin A, Goubau D, Baron-Bodo V, Lin R, Massie B, Salcedo M, Hiscott J. (2006) *Cancer Res.* **66**(21):10576-85.

APPENDICES: none

SUPPORTING DATA: starts next page

FIGURE 1

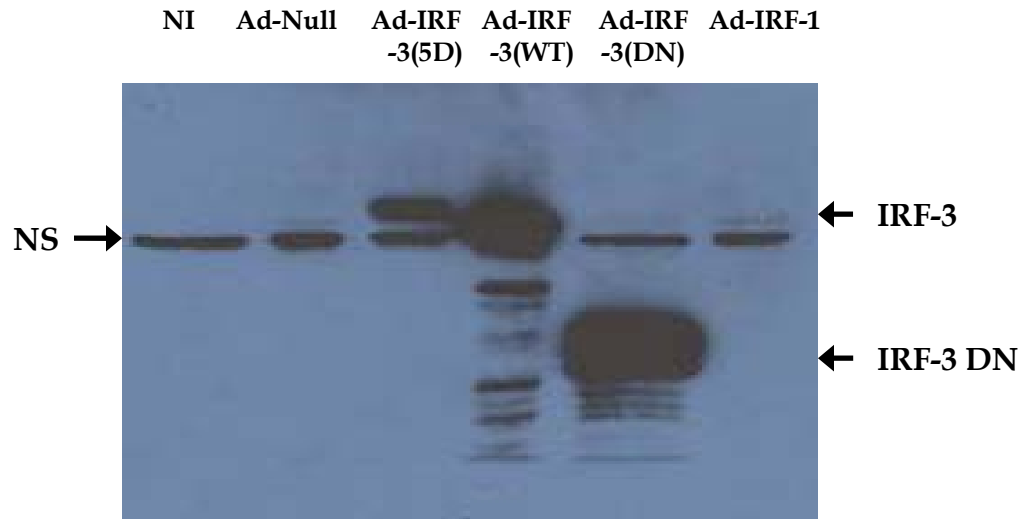


Figure 1. IRF-3 protein assessed by Western blot in C3-L5. Mouse C3-L5 cells were treated as indicated at MOI 100 and 24 hours later cells were harvested and underwent immunoblotting using antibody against human IRF-3. No infection (NI), Ad-Null, and Ad-IRF-1 do not produce IRF-3. Ad-IRF-3(WT) and Ad-IRF-3(5D) produce detectable IRF-3 and IRF-3(DN) produces a smaller detectable fragment which acts as a dominant negative protein. NS = nonspecific binding.

TABLE 1**C3-L5 cells**

	IFN- α (pg/ml)	IFN- β (pg/ml)
NI	5	45
Ad-Null	7	13
Ad-IRF-3(5D)	12	4078
Ad-IRF-3(WT)	724	3968
Ad-IRF-3(DN)	11	53
Ad-IRF-1	518	8

Table 1. IRF-3 produced by Ad-IRF-3(5D) and Ad-IRF-3(WT) are functional. Mouse C3-L5 cells were transduced with indicated adenovirus (NI = no infection, Ad-Null = empty vector) at MOI 100 (MOI needed for >95% transduction in C3-L5 based on green cells seen with Ad-EGFP infection) and media harvested after 24 hours for mouse IFN- α or IFN- β by ELISA.

TABLE 2**MDA468 Cells**

	IFN- α (pg/ml)	IFN- β (pg/ml)
NI	<0.1	281 $\pm$ 4
Ad-Null	<0.1	336 $\pm$ 48
Ad-IRF-3(5D)	<0.1	1262 $\pm$ 153
Ad-IRF-3(WT)	<0.1	243 $\pm$ 26
Ad-IRF-3(DN)	<0.1	256 $\pm$ 3
Ad-IRF-1	717	277 $\pm$ 4

Table 2. IRF-3(WT) does not increase human IFN- α or IFN- β in MDA468 cells. Human MDA468 breast cancer cells were transduced with indicated adenovirus (NI = no infection, Ad-Null = empty vector) at MOI 25 (MOI needed for >95% transduction based on green cells seen with Ad-EGFP infection) and media harvested in 24 hours for human IFN- α and IFN- β .

FIGURE 2

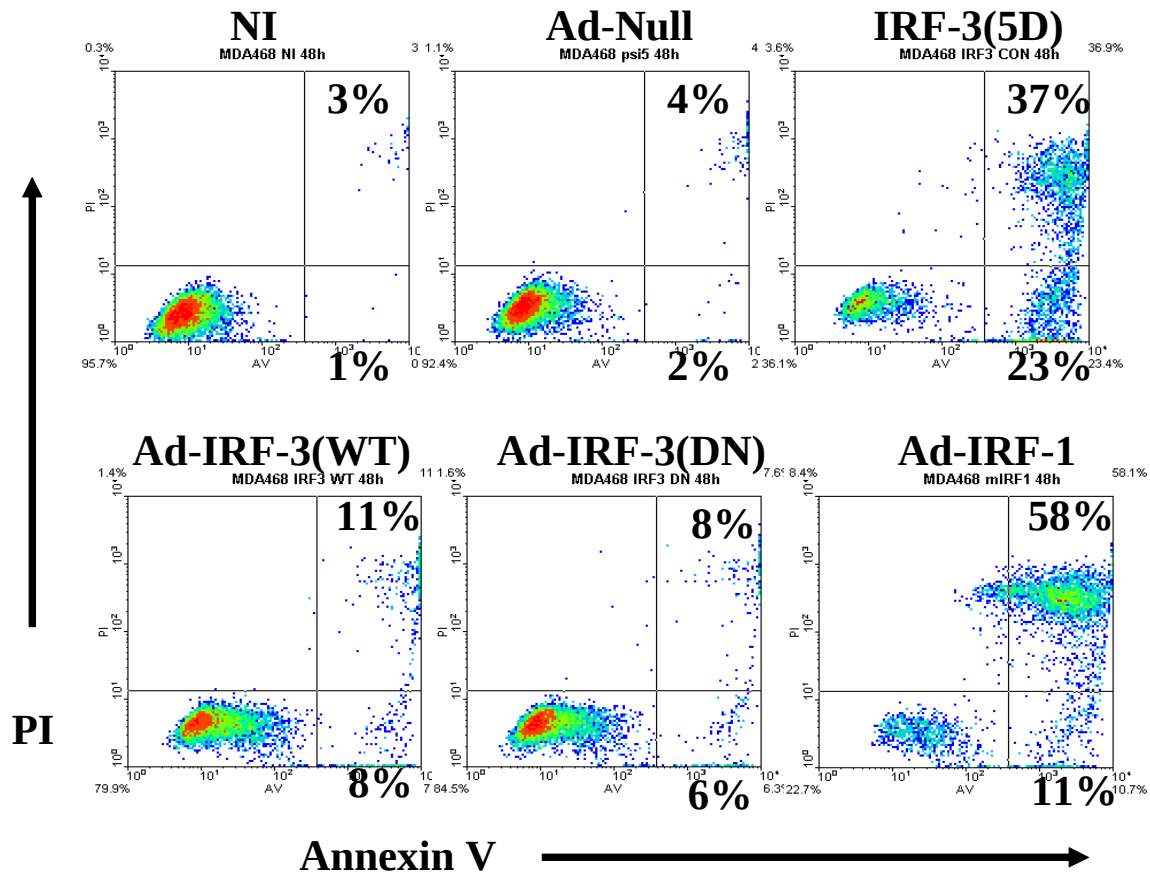


Figure 2. IRF-3 induces apoptosis in MDA468 breast cancer cells. MDA468 cells were transduced with indicated adenovirus at MOI 25 and underwent Annexin V and PI staining as described in the Materials and Methods. Cells underwent flow cytometry resulting in early apoptotic cells in the lower right quadrant and late apoptotic cells in the upper right quadrant. Percent cells in right quadrants are listed.

FIGURE 3

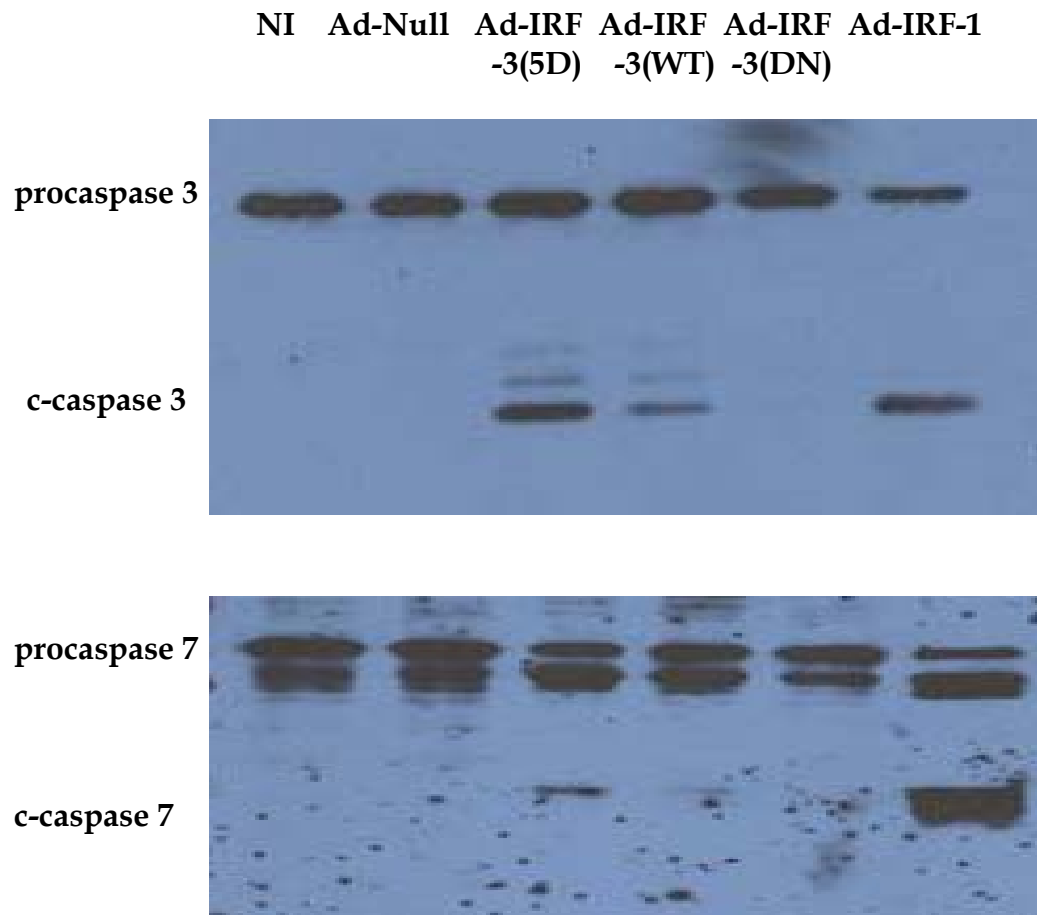


Figure 3. IRF-3 causes caspase-3 and caspase-7 cleavage in MDA468 cells. MDA468 cells were transduced with indicated adenovirus at MOI 25 and cells harvested at 24 hours for immunoblotting with antibody against caspase-3 or caspase-7. Ad-IRF-1 is a positive control for caspase-3 and -7 cleavage.

FIGURE 4

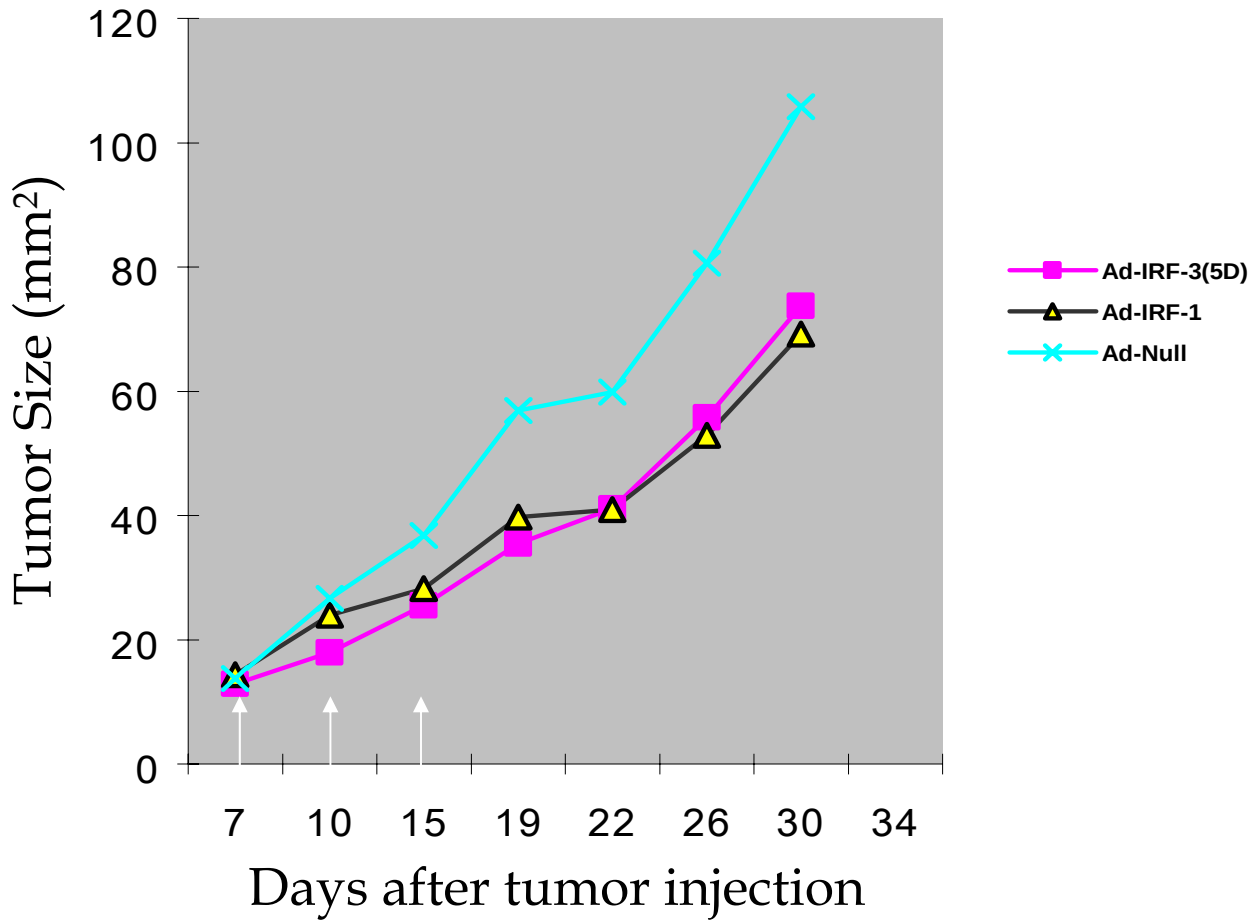


Figure 4. Ad-IRF-3 inhibits breast cancer growth *in vivo*. Intratumoral injection of Ad-IRF-3 into established tumors inhibited tumor growth similar to Ad-IRF-1 versus empty vector Ad-Null control. After injection C3-L5 cells, recombinant Ad were injected 3 times (indicated by arrows) and tumor size measured every 4 days.

FIGURE 5

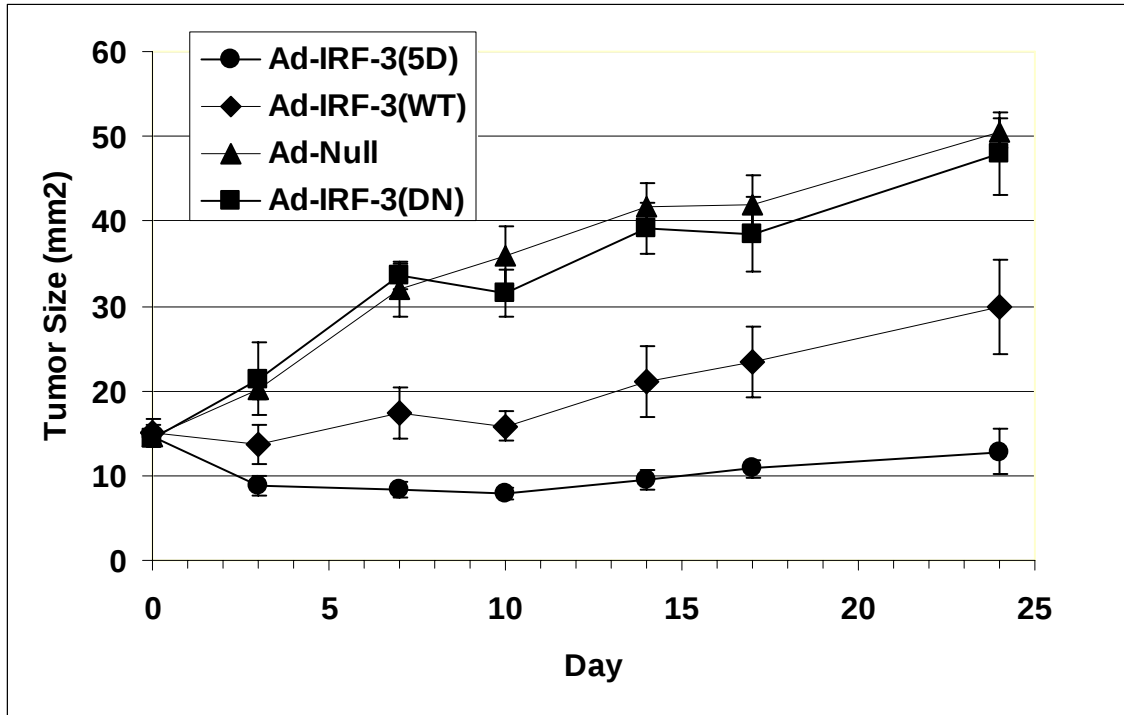


Figure 5. Established MDA468 tumors injected intratumorally with rAd show growth suppression Ad-IRF-3(5D)>Ad-IRF-3(WT)>controls. MDA468 cells were injected in the mammary line of SCID/Beige mice. Established tumors of mice (n=5 per group) were injected with adenovirus or empty vector adenovirus control (Ad-Null) three times in one week. Measurements are given as mean tumor size (width x length) $\pm$ SEM.