# Intravenous Administration of Adenoviruses Targeting Transforming Growth Factor Beta Signaling Inhibits Established Bone Metastases in 4T1 Mouse Mammary Tumor Model in an Immunocompetent Syngeneic Host

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**Abstract**

We have examined the effect of adenoviruses expressing soluble transforming growth factor receptorII-Fc (sTGFβRIIFc) in 4T1 mouse mammary tumor bone metastasis model in syngeneic BALB/c mice. Infection of 4T1 cells with a non-replicating adenovirus Ad(E1-).sTβRFc, or with two oncolytic adenoviruses Ad.sTβRFc and TAd.sTβRFc expressing sTGFβRIIFc produced sTGFβRIIFc protein. Oncolytic viruses were capable of replicating and induced cytotoxicity in 4T1 cells. 4T1 cells were resistant to the cytotoxic effects of TGFBβ-1 (up to 10 ng/ml). However, TGFβ-1 induced the phosphorylations of SMAD2 and SMAD3, which were inhibited by co-incubation with sTGFβRIIFc protein. TGFβ-1 also induced IL-11, a well know osteolytic factor. Intracardiac injection of 4T1-luc2 cells produced bone metastasis by day 4. Intravenous injection of Ad.sTβRFc (on days 5 and 7) followed by bioluminescence imaging of mice on days 7, 11 and 14 in tumor bearing mice indicated the inhibition of bone metastasis progression ($p<0.05$). X-ray radiography of mice on day 14 showed a significant reduction of the lesion size by Ad.sTβRFc ($p<0.01$) and TAd.sTβRFc ($p<0.05$). However, Ad(E1-).sTβRFc expressing sTGFβRIIFc was not effective in inhibiting bone metastases. Thus, systemic administration of Ad.sTβRFc and TAd.sTβRFc can inhibit bone metastasis in 4T1 mouse mammary tumor model, and can be developed as potential anti-tumor agents for breast cancer.

**Introduction**

In the United States, nearly 209,000 women are diagnosed with breast cancer, and about 43,000 die of this malignancy each year.[1] A majority of the women during the advanced stages of breast cancer develop bone metastases, tumor-induced bone destruction, hypercalcaemia and spinal cord compression, thus seriously compromising the life style of the affected patients.[2] Currently, there are only limited therapies for bone metastases. The two types of therapies bisphosphonates, and recently developed denosumab, an antibody against RANKL, though can inhibit bone resorption, their ability to cure bone metastases is not known.[3] Thus development of novel therapies of breast cancer bone metastasis is an unmet need in medicine.

In the recent years oncolytic adenoviruses have shown some potential in the treatment of cancer [4-9], and their utility in the treatment of breast cancer bone metastasis in the animal models is beginning to be explored.[5, 6, 10, 11] In an attempt to develop novel therapy approach for bone metastases, our laboratory has developed oncolytic adenoviruses that would kill the cancer cells, and simultaneously express a soluble form of TGFβ ReceptorII-Fc (sTGFβRIIFc) that can target TGFβ-induced signaling pathways.[11, 12] We chose to target TGFβ pathway, because high levels of circulating TGFβ protein is a poor prognostic marker in breast cancer patients.[13, 14], and aberrant TGFβ signaling at the bone metastasis site has been postulated to be a key factor in the progression of breast cancer bone metastases.[14-18] Because of this, there is a growing interest in developing inhibitors of TGFβ signaling for the treatment of various cancer metastases.[19-23] Using MDA-MB-231 human breast cancer bone metastasis model in immunodeficient mice, we have recently shown that intravenous delivery of oncolytic

adenoviruses - Ad.sTβRFc and TAd.sTβRFc, in tumor bearing mice are effective in inhibiting the established bone metastases.[10, 11] However, before initiating a clinical trial in breast cancer patients, it would be important to examine the efficacy of these oncolytic adenoviruses in an immunocompetent animal model as they have the ability to limit adenovirus replication and thus it's efficacy. Keeping that in mind, we have now conducted *in vitro* and *in vivo* studies using a mouse mammary 4T1 tumor cell model. We report here that infection of 4T1 cells with recombinant adenoviruses produces transgene expression; 4T1 cells support adenoviral replication and are killed by oncolytic adenoviruses. Moreover, although these cells were resistant to TGFβ-1 induced cytotoxicity, TGFβ was able to activate signaling. More importantly, intracardiac inoculation of 4T1 cells in BALB/c mice produces bone metastasis and osteolytic lesions, and thus is an appropriate pre-clinical model for our purpose. We report here that intravenous injections of Ad.sTβRFc and TAd.sTβRFc inhibited the established skeletal metastasis in BALB/c mice. Bbased on our findings we believe that oncolytic adenoviruses targeting TGFβ pathways can be developed for treating breast cancer bone metastasis.

## Materials and Methods

### Cell culture

4T1 (ATCC, Manassas, VA) a mammary tumor cell line, 4T1-luc2 (Caliper life sciences, Hopkinton, MA), MV1Lu (ATCC) mink epithelial cell line, and HEK 293

(ATCC, Manassas, VA) human embryonic kidney cells were grown in DMEM containing 10% bovine calf serum (Invitrogen, Grand Island, NY).

**Adenoviral vectors**

Adenoviral vectors used in these studies are: Ad(E1-).Null, an E1 minus replication-deficient adenovirus containing no foreign gene; Ad(E1-).GFP, a replication-deficient adenovirus expressing eGFP protein; Ad(E1-).sTβRFc, a replication-deficient adenovirus expressing sTGFβRIIFc gene; Ad.sTβRFc, an oncolytic adenovirus expressing sTGFβRIIFc gene (constructed using *dl*01/07 mutant of Ad5, containing two deletions in E1A region as previously described)[24]; and TAd.sTβRFc, an oncolytic adenovirus expressing sTGFβRIIFC gene except human TERT promoter was used to drive the adenoviral replication as published.[12, 25] Adenoviral vectors were grown in HEK 293 cells and purified by double $CsCl_2$ gradient as described [26]. Viral particle (VP) numbers were determined by measuring $OD_{260}$ of the SDS-treated adenoviral solutions.

**Adenoviral replication assay**

Two types of viral replication assays were conducted in 4T1 cells. In one assay 4T1 cells were plated in 6-well dishes ($4x10^5$ cells/well). Next day, cells were incubated with adenoviral vectors for 3 hrs or 48 hrs, and crude viral lysates, were used to infect HEK 293 cells and analyzed by hexon staining assay as described earlier.[25] Viral titers (burst size) were represented by an increase in viral titer from 3 hrs to 48 hrs as described.[25] In a second viral replication assay, 4T1 cells were continuously exposed to viral vectors ($2.5x10^4$ VPs/cell) for 3 days. Cells were then subjected to

immunohistochemistry for adenoviral hexon protein. Hexon expressing brown cells were counted under the microscope to quantify the hexon-positive cells as a function of viral replication.

**Cytotoxicity assays**

To measure TGFβ-1-induced cytotoxicity, cells were plated in 96-well plates ($10^4$ cells/well). Next day, cells were infected with various concentrations of TGFβ-1 (1-10 ng/ml) (Sigma, St. Louis), and the incubations continued for 7 days. Cells were washed, fixed and stained with supfhorhodamine B (SRB) (Sigma, St. Louis), and the $A_{560}$ measured as previously described.[26] Untreated control cells were considered to have 100% survival. To examine viral-induced cytotoxicities, the same protocol was used except 4T1cells were incubated with various doses of adenoviral vectors for 7 days prior to SRB staining.

**sTGFβRIIFc expression**

4T1 cells were plated in 6-well dishes ($4x10^5$ cells/well). Next day, cells were infected with various viral vectors ($2x10^4$ VPs/cell). After 24 hrs, media was changed to serum free media, and the incubations continued for another 24 hrs. sTGFβRIIFc expression in the media and cell lysates were examined by Western blot analyses as previously described.[12] sTGβRIIFc protein amounts in the media were measured by ELISA using antibodies against the human IgG Fcg fragment (Jackson ImmunoResearch) as previously described.[10]

**SMAD phosphorylation**

4T1 cells were plated in 6-well plates ($4x10^5$ cells/well). Next day, cells were serum starved for overnight, and then treated with TGFβ-1 (5 ng/ml) in the absence or presence of sTGFβRIIFc (1μg /ml) for 1 hr. Cells were analyzed for p-SMAD2, p-SMAD3, and for total SMAD2/3 using Western blots as previously described. [27] The blots were visualized by enhanced chemiluminescence substrate (Amersham Biosciences, Piscataway, NJ).

**IL-11 assays**

4T1 cells were plated in 6-well plates ($10^5$ cells per well). Next day, cells were serum starved, and exposed to TGFβ-1 (0.1, 1 or 5 ng/ml) for 48 hrs. Media were analyzed for IL-11 levels by ELISA using previously described method. [27]

**Animal model**

All animal experiments were conducted using animal protocols approved by the IACUC committee of the NorthShore University HealthSystem. To establish bone metastasis, 4T1-luc2 cells were injected in the left heart ventricle (day 0) of 4 weeks old BALB/c mice (Charles River laboratories, Wilmington, MA). On day 4, mice were subjected to BLI in dorsal and ventral position using Xenogen IVIS Spectrum imaging equipment (Caliper life sciences, Hopkinton, MA). Photons signals were quantified using living software 3.0 (Caliper life sciences, Hopkinton, MA) as previously described.[11] Mice were divided in various groups, with statistically indistinguishable BLI signals amongst each group. Various viral vectors were administered via tail vein on day 5 and

on day 7 ($5x10^{10}$ VPs per injection/mouse, each injection in a 0.1 ml volume). Control group of mice were administered with the buffer alone.

**Bioluminescence imaging**

Mice were imaged in dorsal and ventral positions on days 7, 11 and 14 using IVIS Spectrum imaging system (Caliper Life Sciences, Hopkinton, MA) and whole body BLI signals were used to quantify the metastasis as previously described.[11] Signals in the hind limbs were separately quantified to measure the skeletal metastasis as described. [11]

**X-ray radiography**

On day 14 following tumor cells injections, mice were also subjected to X-ray radiography using Faxitron (Faxitron X-ray Corporation, Wheeling, IL). Skeletal lesions sizes were measured in femur and tibia of both the hind limbs using Image J software as described earlier.[10, 11]

**Statistical evaluation.**

All statistical analyses were performed using GraphPad Prizm 5 (GraphPad software, San Diego, CA). Data are presented as mean ± SEM. To analyze, BLI signal progression, a two-way repeated-measure ANOVA followed by Bonferroni post-tests was used. For multiple groups, statistical significance was analyzed using one-way ANOVA followed by Bonferroni post-tests. $p<0.05$ was considered statistically significant difference.

## Results

### 4T1 cells can be infected with human adenoviral vectors

Experiments were conducted to examine the infectability of 4T1 cells with replication-deficient and replication competent adenoviral vectors. 4T1 cells were infected with Ad(E1-).GFP, a non-replicating adenovirus, for 48 hrs and cells visualized under a florescent microscope. Nearly 100% of the cells produced strong GFP signal (Figure 1a). In another experiment, cells were infected with Ad(E1-).sTβRFc, a replication-deficient adenovirus, and two oncolytic adenoviruses- Ad.sTβRFc and TAd.sTβRFc. Cell lysates and the extracellular media were subjected to Western blot analyses for sTGFβRIIFc expression. Infection of 4T1 cells with Ad(E1-).sTβRFc, Ad.sTβRFc and TAd.sTβRFc resulted in sTGFβRIIFc protein production, which could be detected in the cell lysates as well as in the extracellular media (Figure 1b). The amounts of sTGFβRIIFc were quantified in the media using ELISA, and were found to be in the range of 6.21-15.48 μg/ml (Figure 1c). These results indicate that 4T1 calls can be infected with human adenoviruses and that infection with Ad(E1-).sTβRFc, Ad.sTβRFc or TAd.sTβRFc leads to the production of sTGFβRIIFc protein, which is secreted into the media.

### Oncolytic adenoviruses replicate and induce cytotoxicity in 4T1 cells

To examine the cytotoxic effects of viral vectors, 4T1 cells were incubated with various adenoviruses for 7-days, and the cytotoxicity assays performed. Both the oncolytic adenoviruses, Ad.sTβRFc and TAd.sTβRFc induced a dose-dependent cytotoxicity in 4T1 cells (Figure 2a). Based on the $IC_{50}$ values, Ad.sTβRFc and TAd.sTβRFc were about 34.2-fold and 24.0-fold more toxic than non-replicating virus Ad(E1-).Null respectively. In contrast, a non-replicating virus Ad(E1-).sTβRFc did not show higher toxicity compared to Ad(E1-).Null (Figure 2a, b).

Next, we examined the replication potential of adenoviral vectors in 4T1 cells. Cells were infected with various viral vectors for 3 hrs, the unbound viruses were washed away and the incubation continued for 48 hrs. Crude cell lysates from 3 hr and 48 hr samples were prepared and the viral titers estimated. Interestingly, in this assay there was no detectable increase of viral burst sizes from 3 hrs to 48 hrs of any of the tested adenoviruses (data not shown). Considering that exposure of 4T1 cells to oncolytic adenoviruses was able to induce cytotoxicity, we were interested to examine if the continuous incubation of 4T1 cells with oncolytic viruses were able to produce significant viral titers that could cause cytotoxicity. To examine this, 4T1 cells were incubated with various adenoviral vectors ($5x10^4$ VPs/cell) at 37º for 72 hrs. Under these conditions, Ad.sTβRFc produced viral titer (Figure 2c) which was about 270-times higher than the non-replicating adenovirus Ad(E1-).Null ($p<0.001$) (Figure 2d). TAd.sTβRFc also produced 147-times higher viral titers compared to the Ad(E1-).Null ($p<0.01$) (Figure 2c, d). However, there was no significant increase in the viral titer of the Ad(E1-).sTβRFc compared to the Ad(E1-).Null. From these results we conclude that continuous

incubation of 4T1 cells with oncolytic adenoviruses can produce viral production in 4T1 cells resulting in the cytotoxicity in the 4T1 cells.

**4T1 cells are resistant to killing by TGFβ, but retain TGFβ-mediated signaling pathways**

Next, we investigated the killing effect of TGFβ in 4T1 cells. 4T1 cells were exposed to various concentrations of TGFβ-1, and 7-days later cytotoxicity was measured. As a positive control, another rodent cell type MV1Lu, known to be sensitive to TGFβ was used. As shown in Figure 3a, there was no or only minimum cytotoxic effect of TGFβ-1 even at the highest concentration used (10 ng/ml) in 4T1 cells, while MV1Lu cells were killed by a very low concentrations of TGFβ-1, with an $IC_{50}$ of less than 0.1 ng/ml.

To examine if TGFβ could induce signaling in 4T1 cells, we exposed these cells to TGFβ-1 and analyzed the cell lysates for SMAD2 and SMAD3 phosphorylation. Figure 3b shows that TGFβ-1 induced SMAD2 and SMAD3 phosphorylation in 4T1 cells. Co-incubation of sTGFβRIIFc with TGFβ-1 inhibited TGFβ-1-dependent SMAD2 and SMAD3 phosphorylation. We also examined the effect of TGFβ-1 on IL-11 production, a known osteolytic factor in human breast cancer cells.[28] TGFβ-1 induced IL-11 protein production in a dose dependent manner (Figure 3c). These results indicate that 4T1 cells respond to TGFβ-1 undergo activation of signaling pathways that are known to favor bone metastases in human breast cancer cells[18, 27, 29], and that sTGFβRIIFc is able to abolish the TGFβ signaling.

**Oncolytic adenoviral-mediated inhibition of 4T1-induced metastases: BLI analyses**

Next, we examined the effect of systemic administration of adenoviral vectors expressing sTGFRIIFc in a 4T1 bone metastasis model. 4T1-luc2 cells were inoculated into left heart ventricle of BALB/c mice. After four days, mice were subjected to whole body bioluminescence imaging (BLI) in both dorsal and ventral positions. Mice were split into multiple groups, with nearly equal BLI signal amongst each group. Two doses of adenoviral vectors were given via tail vein- first dose on day 5 ($5x10^{10}$ VPs/mouse), and a second dose on day 7 ($5x10^{10}$ VPs/mouse). Mice were subjected to BLI on days 7, 11 and 14 following tumor cell injection. A representative mouse showing BLI signal from each treatment group is shown in Figure 4a. Whole body BLI signals were quantified and are shown in Figure 5a. There was a time-dependent increase in the whole body BLI signal to $0.88x10^{11}$ photons/sec in the control group of mice that received buffer alone (Figure 4b). There was no significant inhibition of BLI signal in the Ad(E1-).Null, Ad(E1-).sTβRFc and TAd.sTβRFc treatment groups (Figure 4b). However, Ad.sTβRFc induced a significant inhibition ($p<0.05$) of whole body BLI. Since 4T1 cells also established bone metastasis in the hind limbs (Figure 4a), the effect of viral vectors on the BLI signal in the hind limbs was quantified. In the control group of mice, the BLI signal in the hind limbs reached to $0.86x10^{10}$ photons/sec, and there was a significant inhibition of the BLI signal accumulation in the hind limbs in Ad.sTβRFc treated group ($p<0.05$). However, Ad(E1-).Null, Ad(E1-).sTβRFc and TAd.sTβRFc treatments had no significant effect in the BLI signal intensity in the hind limbs (Figure 4c).

**Oncolytic adenoviral vectors'-mediated inhibition of 4T1-induced metastases: X-ray analyses**

To further examine the effects of vectors' administration on bone metastases, mice were subjected to X-ray radiography on day 14. A representative example of X-ray radiographs from each group is shown in Figure 5a. The long bones in buffer treated and Ad(E1-).Null treated had large osteolytic lesions, as indicated by red arrows. The lesion sizes were relatively smaller in the other treatment groups. The tumor lesions in the hind limbs were quantified using Image J program and are shown in Figure 5b. Tumor sizes in the buffer groups were 6.92±1.27 $mm^2$. Tumor sizes in Ad(E1-).Null, Ad(E1-).sTβRFc, Ad.sTβRFc and TAd.sTβRFc treatment groups were 5.79±0.86, 3.56±0.72, 2.30±0.69, and 2.94±0.57 $mm^2$ respectively. These results indicate no significant effect on the tumor sizes by Ad(E1-).Null, Ad(E1-).sTβRFc, but a significant effect by the replicating viruses- Ad.sTβRFc ($p < 0.01$) and TAd.sTβRFc ($p < 0.05$) (Figure 6b).

While, we were able to monitor BLI and X-ray till day 14, by day 18 a number of animal deaths were observed in each of the treatment groups. In the buffer group (3/9), in the Ad(E1-).Null group (4/9), in the Ad(E1-).sTβRFc group (3/9), in th eAd.sTβRFc group (2/11), and in TAd.sTβRFc group (3/11) mice died between day 14 and day 18. To confirm the sTGFβRIIFc expression in the blood, blood samples were collected from the remaining mice on day 18, and the sTGFβRIIFc production analyzed by ELISA. The results shown in Figure 5c indicate high levels- 2.43±1.67 μg/ml, 6.53±16.31 μg/ml, 15.41±24.86 μg/ml of sTGFβRIIFc in blood samples from Ad(E1-).sTβRFc, Ad.sTβRFc and TAd.sTβRFc treatment groups, respectively. Thus, while Ad(E1-).sTβRFc infection

resulted in sTGFβRIIFc production, it appears that both the replicating viruses expressing sTGFβRIIFc were more effective in inhibiting bone metastases.

**Discussion**

The key finding here is that intravenous delivery of oncolytic viruses Ad.sTβRFc and TAd.sTβRFc expressing sTGFβRIIFc can inhibit bone metastasis in 4T1 mouse mammary tumor bone metastasis model in a syngeneic host. Most of the previously published studies using oncolytic adenoviruses have been conducted in human xenografts established in immunodeficient nude mice, mainly because mouse tumor cells are not considered good targets for the human adenoviruses. However, it is critical that we continue to explore the animal models in which oncolytic adenoviruses can be examined in immunocompetent syngeneic host as described here. This is critical to ensure that host immune response against the adenovirus will not limit the therapeutic potential of adenovirus. It is quite interesting that though 4T1 cells can be infected with human adenoviruses resulting in high level transgene expression indicating the presence of adenoviral receptors even in mouse 4T1 cells. However, unlike human breast cancer cells, if adenoviruses are first bound with the 4T1 cells for short duration (3 hrs), and the excess of viral particles are washed away, no virus replication was detectable. Because of such observations, it is generally believed that human adenoviruses cannot replicate in the mouse 4T1 cells. However, our finding here that continuous exposure of 4T1 cells with oncolytic adenoviral vectors can result in virus entry and replicationclearly demonstrate that human adenoviruses can indeed infect mouse tumor cells perhaps via an unknown receptor.. While the exact mechanism of the Ad.sTβRFc and TAd.sTβRFc-

induced toxicity in 4T1 tumor model remains to be examined, it is tempting to speculate that the viral replication resulting in the cytotoxicity of mouse tumor cells could play a role in mediating the *in vivo* anti-tumor responses being reported here.

Another important observation is the inability of TGFβ to kill 4T1 cells and yet induce TGFβ signaling pathway (SMAD-phosphorylation), and the production of IL-11 (a well known osteolytic factor in human breast cancer bone metastasis). Thus, in this regard 4T1 is an appropriate tumor model for examining the role of TGFβ signaling in bone metastases. In the radiographic analyses, a non-replicating adenovirus expressing sTGFβRIIFc showed some inhibition of bone metastasis, albeit weaker than oncolytic adenoviruses expressing sTGFβRIIFc. Again, these studies suggest that while sTGFβRIIFc expression plays a critical role in mediating the inhibition of bone metastasis as previously shown, perhaps it is the combination of sTGFβRIIFc expression and viral-mediated cytotoxicity that could be potentially playing a role in mediating inhibition of bone metastasis. In addition to understanding the role of viral replication and the inhibition of TGFβ signaling at the tumor-bone microenvironment, the availability of this tumor model will also allow us to explore the role of innate and humoral immune response[30-32]; and the role of TGFβ in suppressing the immune system[14, 15, 33] and how that can be reversed by the oncolytic adenoviruses expressing sTGFβRIIFc. These questions can be addressed only in fully immune competent animal models.

In conclusion, our work described here shows that oncolytic adenoviruses targeting TGFβ pathway can inhibit breast cancer bone metastases in a mouse mammary tumor model established in a syngeneic immunocompetent host, and represents an important step in developing oncolytic adenoviruses for the treatment of breast cancer

bone metastases. Moreover, this animal model will now allow us to investigate the underlying molecular mechanism of action, which may help in further refining this method of treatment.

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**Figure Legends**

**Figure 1.** Adenoviral-mediated transgene expression in 4T1 cells. (**a**) 4T1 cells were infected with Ad(E1-).GFP ( $2.5 \times 10^4$ VPs/cell) for 24 hrs. Cells were photographed (100x) using a florescent microscope. Same viewing fields were used to take phase contrast (left) or florescent (right) images. (**b**) 4T1 cells were infected with various adenoviral vectors ($2.5 \times 10^4$ VPs/cell). Cell lysates and the media were analyzed by Western blots for sTGFβRIIFc protein expression. (**c**) Extracellular media were used to examine sTGFβRIIFC levels by ELISA method.

**Figure 2.** Adenoviral replication and cytotoxicity in 4T1 cells. (**a**) 4T1 cells were exposed to various viral vectors for 7-days. The cytotoxicity assays were conducted by sulforhodamine B staining. Control cells were considered 100% survival. (**b**) IC50s for each virus were calculated, and the IC50 ratios between each vector and Ad(E1-).Null are shown. (**c**) 4T1 cells were infected with various viral vectors for 72 hrs, and stained for hexon protein to measure the viral titers. (**d**) The ratios between the viral titer of each virus and that of Ad(E1-).Null are shown. *** $p<0.001$, ** $p<0.01$.

**Figure 3.** TGFβ-1 effects and TGFβ signaling pathways in 4T1 cells. (**a**) 4T1 or MV1Lu cells were exposed to various concentrations of TGFβ-1. After 7-days, cytotoxicity assays were conducted using sulphorhodamine staining. Control cells were considered to be 100% survival. (**b**) 4T1 cells were exposed to TGFβ-1 (5 ng/ml) for 60 minutes in the absence or presence of sTGFβRIIFc (1 μg/ml). Cell lysates were examined for p-SMAD3

and total SMAD2/3 by Western blot analysis. (**c**) 4T1 cells were exposed to various concentrations of TGFβ-1 for 48 hrs. Cell media were used to measure IL-11 levels by ELISA method.

**Figure 4.** Effect of systemic delivery of viral vectors on 4T1 bone metastasis: BLI analysis. 4T1-luc2 cells were injected in BALB/c mice ($5x10^4$ cells/mouse) on day 0. Initial BLI was performed on day 4; mice with positive tumors were administered viral vectors or buffer (via tail vein) on day 5 and day 7. BLI were conducted on days 7, 11 and 14. Number of mice in various treatment groups were- buffer (n=9), Ad(E1-).Null (n=9), Ad(E1-).sTβRFc (n=9), Ad.sTβRFc (n=11) and TAd.sTβRFc (n=11). Representative mice in each treatment groups are shown. (**b**) BLI signal in the whole body of mice in various treatment groups were quantified and are shown. (**c**) To measure bone metastases, BLI signals in the hind limbs (shown by red circles) were quantified in each treatment groups and are shown. * $p<0.05$.

**Figure 5.** Effect of systemic delivery of viral vectors on 4T1bone metastasis: X-ray radiography. (**a**) Mice form the above experiment described in Figure 5, were subjected to X-ray radiography on day 14. (**b**) Lesion sizes in each mouse were calculated using Image J software. Results shown are the average lesion sizes in the hind limbs in each of the treatment groups. * $p<0.05$, ** $p<0.01$.

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