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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

We proposed to determine whether overexpression of survivin results in radioresistance and the possible mechanisms; whether irradiation downregulates $p34^{CDC2}$ and its mechanism in vascular endothelium and whether CDK (cyclin dependent kinase) inhibitors sensitize it to radiation injury; the mechanism of survivin deregulation in breast cancer and whether inhibition of survivin or its regulator, $p34^{CDC2}$ abolishes radioresistance. We found that overexpression of survivin leads to radiation resistance and CDK inhibitors result in radiosensitization. We also found that deregulation of survivin in breast cancer is mediated by Stat3 (Signal transducer and activator of transcription). Co-inhibition of survivin and Stat3 results in significantly increased sensitization of breast cancer. In the next two years, we will determine the biological significance of combining survivin inhibitors or CDK inhibitors with radiotherapy in xenograft models of breast cancer. The proposed study will address the biological basis of combining radiotherapy with inhibitors of survivin or CDK in the treatment of locally advanced breast cancer.

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1. Introduction:

We have proposed to determine whether overexpression of survivin results in radioresistance and the possible mechanisms. We intend to determine whether irradiation downregulates $p34^{CDC2}$ and its mechanism in vascular endothelium and whether CDK inhibitors sensitize it to radiation injury. We proposed to determine the mechanism of survivin deregulation in breast cancer and whether inhibition of survivin or its regulator, $p34^{CDC2}$ abolishes radioresistance. Finally, we want to determine the biological significance of combining survivin inhibitors or CDK inhibitors with radiotherapy in xenograft models of breast cancer. The proposed study will address the biological basis of combining radiotherapy with inhibitors of survivin or CDK in the treatment of locally advanced breast cancer.

2. Body:

To accomplish the proposed studies: we have the following Statement of Work for the first 12 months:

Task 1. To determine whether overexpression of survivin results in radioresistance and the possible mechanisms (Months 1-9):

We have shown survivin overexpression leads to radiation resistance as demonstrated in reference 1.

Task 2. To determine whether irradiation downregulates p34CDC2 and its mechanism in vascular endothelium and whether CDK inhibitors sensitize it to radiation injury. (Months 9-19):

Our preliminary data showed that inhibitors of survivin and CDK result in synergistic radiosensitization.

Task 3. To determine the mechanism of survivin deregulation in breast cancer and whether inhibition of survivin or its regulator, p34CDC2 abolishes radioresistance. (Months 20-28):

We found that deregulation of survivin in breast cancer is mediated by Stat3. These data will be presented at the 4th Era of Hope meeting.

Task 4. To determine the biological significance of combining survivin inhibitors or CDK inhibitors with radiotherapy in xenograft models of breast cancer (Months 29-36)

These experiments are being planned.

Results Obtained: Please see the attached results.

3. Key Research Accomplishments:

1. Survivin overexpression leads to radioresistance.
2. Inhibitors of survivin and CDK result in radiosensitization.
3. Stat3 mediates deregulation of survivin in breast cancer.

4. Reportable outcomes:

1. Kwang Woon Kim and Bo Lu. Stat3 mediates transcriptional downregulation of survivin following irradiation. (submitted to Cancer Research). Poster presentation at the Era of Hope meeting (Philadelphia, June 2005).

Abstract: Signal transducer and activator of transcription 3 (STAT3), which was identified as an interleukin-6- activated transcription factor, transduces signaling from the cytoplasm to the nucleus and then

activates expression of many targeted genes. It plays an important role in controlling cell transformation and oncogenesis. In previous studies, we found that radiation (3Gy) significantly reduced both protein and mRNA levels of survivin in human umbilical vein endothelial cells (HUVECs), but not in tumor cell lines. In this study, we examined whether Stat3 mediates survivin expression following irradiation in HUVECs and breast cancer cells. To determine if Stat3 regulated survivin expression in HUVECs, we infected adenovirus vector (pAdCMVpLpA(-)loxp-SSP), adenovirus Stat3-C, or adenovirus antisense Stat3-C into HUVECs. The protein level of survivin was significantly increased by the expression of adenovirus Stat3-C. The levels of phospho-Tyr 705 Stat3 following irradiation was reduced in HUVECs, but not phospho-Tyr 727 Stat3. To conform if radiation specifically reduced phospho-Tyr 705 Stat3, we treated EGF to HUVECs. Radiation interfered with EGF-induced tyrosine phosphorylation (Tyr 705) of Stat3 in the HUVECs. In the MDA-MB-231 and MCF-7 breast cancer cells, radiation (3Gy) did not decrease levels of both phospho-Tyr 705 Stat3 and phospho-Tyr 727 Stat3. Stat3 DNA- binding activity following irradiation was specifically downregulated at 30min in HUVECs. Cell survivial as measured by clonogenic assay suggested that Stat3 ASO mixed with Survivin ASO resulted in the best radiosensitization of both MDA-MB-231 and MCF-7 breast cancer although Survivin ASO itself showed strong radiosensitizing activity. These results suggest that STAT3 is a survivin modulator and may be a therapeutic target for radiation sensitization in breast cancer.

2. Carolyn Cao, Mu Yi, Dennis E. Hallahan, Bo Lu. Radiation sensitization of lung cancer through inhibition of survivin and XIAP. Oncogene. 2004 Sep 16;23(42):7047-52.

5. Conclusions: We have found that deregulation of survivin in breast cancer is mediated by Stat3. This could lead to radioresistance. Inhibitors of surviving, CDK and Stat3 enhance therapeutic effects of radiation. We will investigate biological consequences of these inhibitors in animal models.

6. Recent Publications that acknowledged this funding:

1. Kwang Woon Kim and Bo Lu. Stat3 mediates transcriptional downregulation of survivin following irradiation. (submitted to Cancer Research).

I). Regulation of Survivin by STAT3.

To determine whether STAT3 regulates the expression of survivin in HUVEC cells, we transduced HUVECs with adenovirus vector (pAdCMVpLpA(-)loxp-SSP) or adenovirus overexpressing antisense STAT3-C as a negative control. Adenoviruses overexpressing constitutively active STAT3-C, or DN STAT3 (Y705F) were used to increase or to decrease Stat3 activity, respectively. Fig. 1 shows the autoradiograph of Western immunoblots. Survivin protein level was markedly elevated in the cells following overexpression of STAT3-C. However, survivin was not induced in the cells infected with negative controls. DN STAT 3(Y705F) reduced the level of phospho-Stat3 Tyr 705 as well as the expression of survivin.

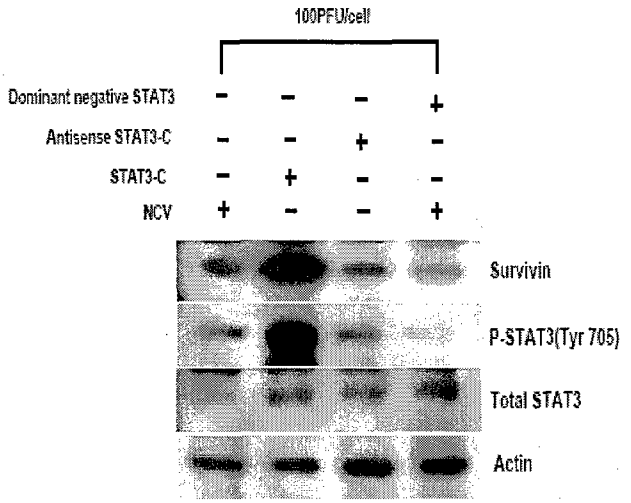


Figure 1. STAT3 regulated survivin protein in HUVECs. HUVECs were transduced with adenoviral vector control (NCV), adenoviruses overexpressing STAT3C, antisense STAT3C or DN STAT3 (Y705F). After 24h of infection, the cells were harvested and fifty microgram total proteins per lane were loaded on 15% SDS-PAGE and subjected to Western blot analysis using various antibodies. Actin was probed to demonstrate equal loading.

II). Irradiation attenuates the active form of phospho-Tyr 705 STAT3.

To examine whether irradiation affects the activation of STAT3, HUVEC, MDA-MB-231 and MCF-7 breast cancer cells were irradiated with 3Gy and harvested at 0, 30, 60, and 240min. Western blot analysis was performed using antibodies against phospho-Tyr 705 STAT3, phospho-Ser 727 STAT3 and total STAT3. As shown in Fig. 2A, phospho-Tyr 705 STAT3 was reduced at 30min and slowly recovered at 240min in HUVECs. However, irradiation did not change the level of phospho-Ser 727 STAT3 in HUVECs. (Fig 2B) To determine whether irradiation affects STAT3 activation by growth factors, we examined the effect of irradiation on STAT3 phosphorylation following pre-treatment of HUVEC cells with epidermal growth factor (EGF). Since STAT3 is activated by EGF signaling (22), HUVEC cells were pre-treated with EGF prior to be subject to either 0 or 3Gy. Stimulation with EGF resulted in an increased level of phospho-Tyr 705 STAT3 whereas irradiation following the EGF treatment reduced the phosphorylation of Tyr 705 STAT3. However, phospho-Ser 727 STAT3 levels showed no change following either EGF or irradiation (data not shown).

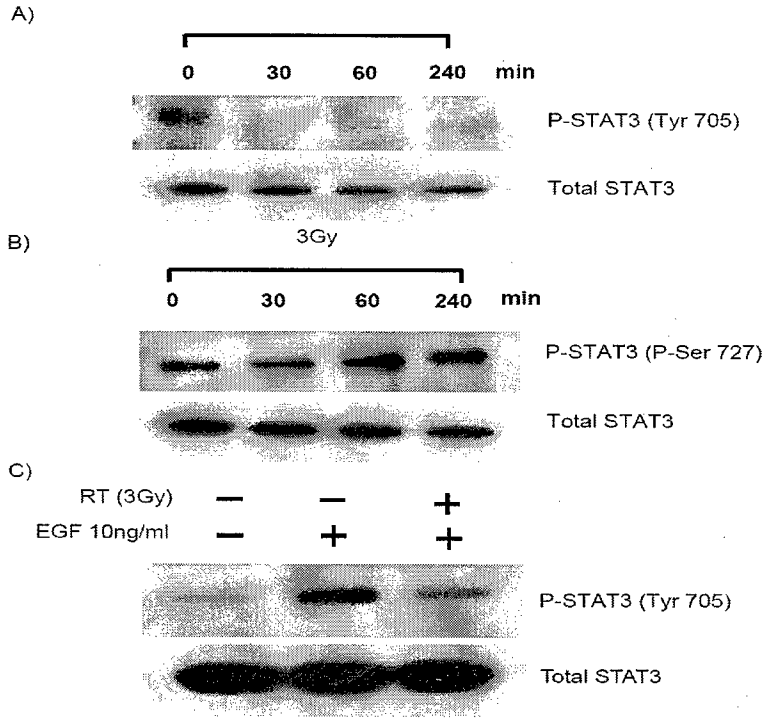
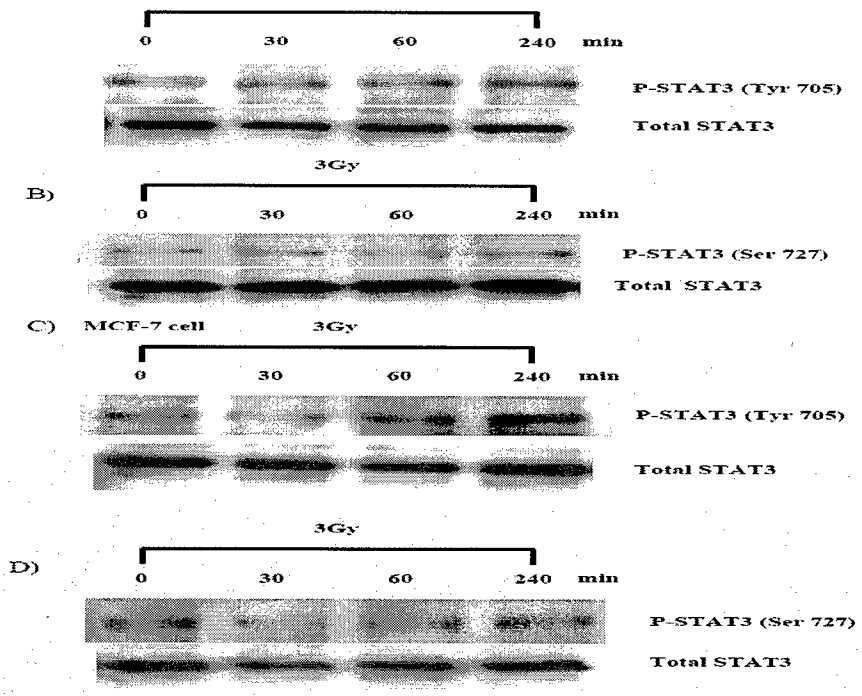


Figure 2. Radiation reduced phospho-Tyr 705 STAT3, not phospho-Ser 727 STAT3 in HUVECs.
HUVECs cells were irradiated with 3Gy. Fifty microgram total proteins per lane were immunoblotted for p-STAT3. (Tyr 705 and Ser 727) and total STAT3. A: p-STAT3 (Tyr705), B: p-STAT3 (Ser 727) C: Radiation interfered with EGF-induced tyrosine phosphorylation (Tyr 705) of STAT3 in the HUVECs.

In the breast cancer cells, both phospho-Tyr 705 STAT3 and phospho-Ser 727 STAT3 levels were not significantly altered by irradiation. (Fig.3)

Figure3. Radiation does not affect STAT3 in breast cancer cells. MDA-MB-231 and MCF-7 breast cancer cells were irradiated with 3Gy. Total cell lysates were extracted at indicated time points. Fifty microgram total proteins per lane were immunoblotted for p-STAT3 (Tyr 705 and Ser 727) and total STAT3. A: MDA-MB-231, p-STAT3 (Tyr705) B: MDA-MB-231, p-STAT3 (Ser 727) C: MCF-7, p-STAT3 (Tyr705) D: MCF-7, p-STAT3 (Ser727).



III). Radiation reduced DNA binding activity of STAT3 in HUVECs:

To determine whether dephosphorylation of STAT3 affects DNA-binding activity, we irradiated HUVECs with 3 Gy. STAT3 DNA binding activity was determined via electrophoretic mobility shift assay (EMSA) using 10ug nuclear protein and a 32-P-labeled oligonucleotide probe containing a consensus-binding motif for STAT3. As shown in Fig. 4, STAT3 activity was reduced at 30min and had slight recovery at 240 min after irradiation. Consistent with Western blot analysis of the attenuated phospho-Tyr 705 STAT3 (Fig.2A), this result also suggests that irradiation inhibited DNA binding activity of STAT3 in HUVEC cells.

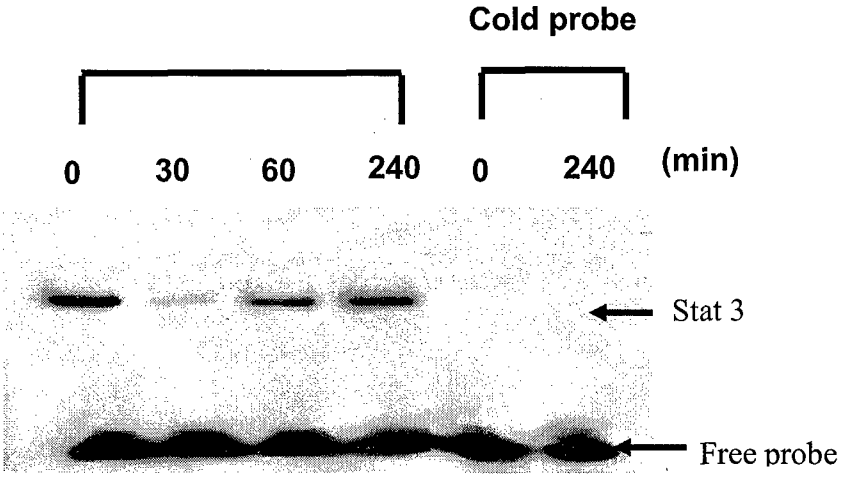


Figure 4. Radiation reduced DNA binding activity of STAT3 in HUVECs.
HUVEC cells were irradiated with 3Gy. Nuclear extracts were prepared at indicated time points. 5ug of nuclear extracts were used for mobility shift assay. Competitor assay was also performed using specific cold probe.

IV). Mutation of Tyr 705 in STAT3 abolished radiation-induced downregulation of survivin:

To determine whether dephosphorylation of STAT3 Tyr 705 is essential for radiation-induced downregulation of survivin, we obtained human mammary epithelial cells overexpressing either wild type STAT3 or Y705F STAT3 mutant from our collaborator. As shown in Figure 5, survivin protein level decreases in wild type cells whereas it remained unchanged in mutant cells following irradiation. This result suggests that Tyr 705 of STAT3 is essential for radiation-mediated downregulation of survivin.

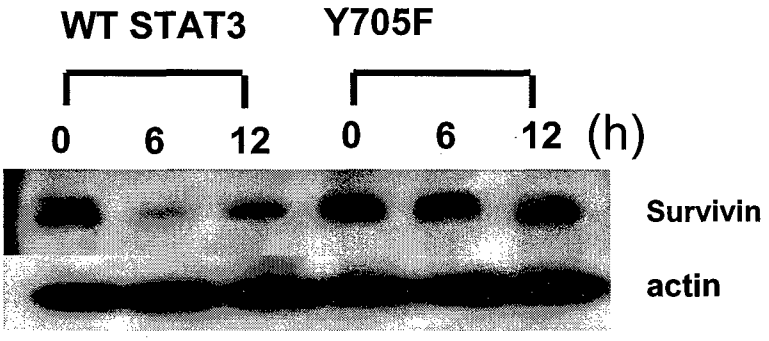
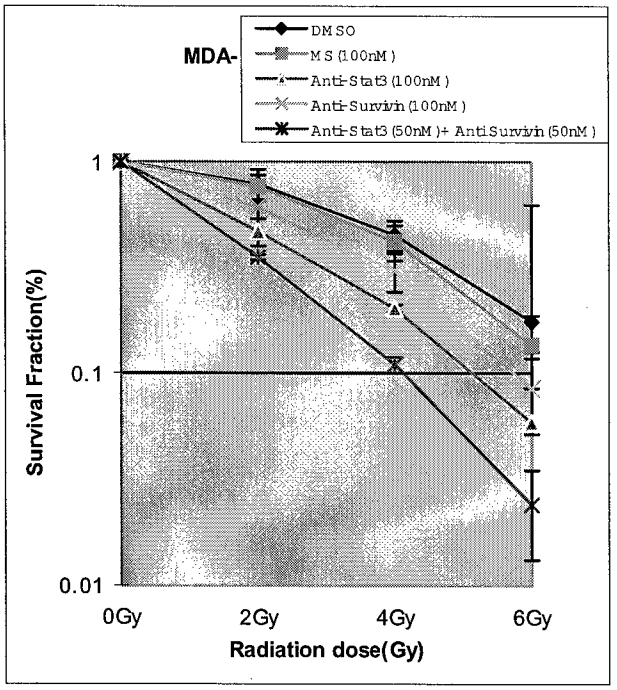


Figure 5. Tyr 705 of STAT3 is essential for radiation-induced downregulation of survivin.
HME1 cells stably transfected with WT STAT3 or Y705 STAT3 expression plasmids were treated with 3Gy. Protein extracts were collected at 0, 6 and 12hr following irradiation. Survivin and beta-actin were probed by western analyses.

V). Combined inhibition of STAT3 and survivin increases radiation sensitization in MDA-MB-231 and MCF-7 breast cancer cells.

To determine whether combined inhibition of both STAT3 and survivin sensitizes MDA-MB-231 and MCF-7 breast cancer cells to radiotherapy, clonogenic assays were performed and are shown in Figure 6. As shown, cells transfected with ASO against STAT3 or ASO against survivin had less surviving colonies as compared to the cells transfected by the control oligo (MS). Also, inhibition of STAT3 resulted in less survival than inhibition of survivin. Furthermore, STAT3 ASO combined with survivin ASO showed the greatest radiosensitization of both MDA-MB-231 and MCF-7 breast cancer cells, although STAT3 ASO alone also showed strong radiosensitizing activity. Therefore, these results suggest that STAT3 and survivin could be important targets for enhancing radiosensitization in breast cancer cells.

A). MDA-MB-231



B). MCF-7

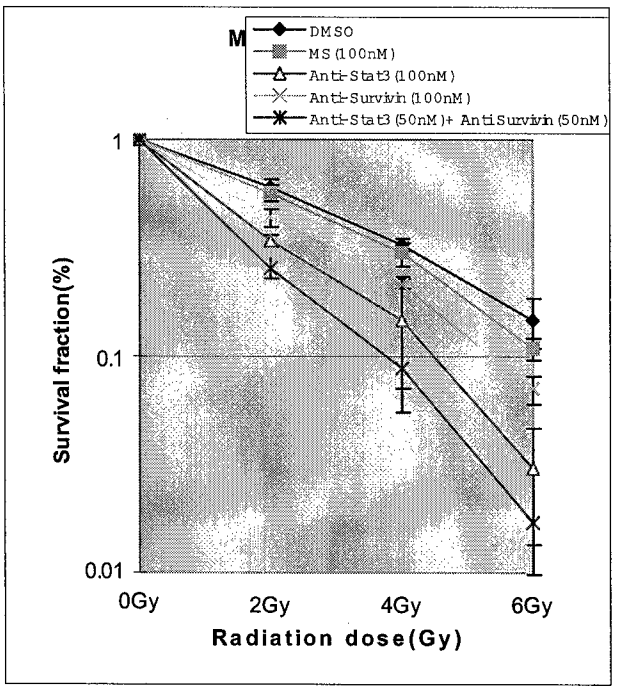


Figure 6. Combined inhibition of STAT3 and survivin increases radiation sensitization in breast cancer cells

MDA-MB-231 and MCF-7 breast cancer cells were transfected with either nothing or MS control oligo (100mM) or ASO survivin (100mM), ASO STAT3 (100mM) ASO STAT3 (50mM) mixed with ASO survivin (50mM). They were then treated with 0, 2, 4 or 6 Gy. After 10 days colonies were stained and scored. Data is shown as the mean +/- SD.