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13. ABSTRACT (Maximum 200 Words)

DOD Award number DAMD17-03-1-0484, "The Role of c-Src Activation in Prostate Tumor Progression", has as its goal an understanding of the mechanisms of how activation of the protein tyrosine kinase, Src, contributes to prostate tumor progression and how Src regulation in host osteoclasts regulates metastatic growth of prostate tumor cells in the bone. In the first year of work, we have successfully developed cell lines from PC3 human prostate tumor cells that are constitutively increased in Src expression, or in which Src expression is induced by doxycyclin. Using these cells, we have demonstrated that increased Src activity leads to increased expression of vascular endothelial growth factor (VEGF); a novel finding implicating Src in promotion of angiogenesis by prostate tumor cells. We have further demonstrated that increased Src expression leads to increased tumorigenic growth in nude mice. Thus, we have provided new evidence that Src activation is important to prostate tumor progression. To examine the role of Src in growth of prostate tumor cells in the bone, we have begun to develop a Src-/- nu/nu strain of mice. Breeding of these mice is underway and two generations of mice have been obtained. Continued studies should provide new insights into role of Src in both the tumor and host environment in promoting prostate tumor cell metastasis.

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INTRODUCTION

DOD Award number DAMD17-03-1-0484, "The Role of c-Src Activation in Prostate Tumor Progression", has as its goal an understanding of the mechanisms of how activation of the protein tyrosine kinase, Src, contributes to prostate tumor progression and how Src regulation in host osteoclasts regulates metastatic growth of prostate tumor cells in the bone. Many epigenetic events contribute to prostate tumor progression, including increased expression of growth factor receptors. When overexpressed, growth factor receptors often constitutively associate with c-Src, resulting in Src activation. Activated Src then contributes to many tumor functions, including resistance to apoptosis and increased expression of angiogenic factors (reviewed by Summy and Gallick, 2003). In prostate cancer cells, we have demonstrated that this activation correlates with increased Vascular Endothelial Growth Factor (VEGF) expression (Kim et al., 2003). However, understanding which functions in tumor progression require Src activation, and the mechanism(s) by which Src contributes to these processes remains unknown, and is a focus of the studies for which this grant was awarded. To complete the tasks we originally proposed, development of prostate tumor cell lines with increased and/or decreased Src expression was required. As discussed below, we have been successful in this endeavor, and have proceeded to finish most of Task 1 (analysis of VEGF expression). Task 2 (resistance to apoptosis) will proceed now that the required cell lines have been generated. Task 3 was to examine tumorigenicity in a Src +/+ and Src-/- background. We have begun analysis in the Src +/+ background, as described below. The rationale for use of the Src-/- background is that, in addition to its role in tumor cells, Src plays a pivotal role in osteoclast function, with Src-/- mice suffering from osteopetrosis, due to defective osteoclasts failing to properly mediate bone remodeling (Soriano et al., 1991). Thus, the other goal of studies in this grant is to determine tumorigenicity in this background. To complete Task 3, we will be required to breed the Src-/- mice into an nu/nu background, exactly as originally proposed. This is necessary because immunocompetent mice will reject human tumor cells. As described below, our breeding program is well under way. Thus, we have made substantial progress in the first year of funding for this grant.

BODY

Task 1

The initial part of Task 1 was to determine the effects of c-Mt and PTEN/MMAC on Src activity and VEGF expression. These studies have been completed, some of them prior to the grant funding beginning. We determined that decreased c-Met expression resulted in decreased Src activation and decreased VEGF expression (Kim et al., 2003); whereas ectopic expression of PTEN/MMAC had no effect on Src activity and no effect on VEGF expression.

Next, we used pharmacologic inhibitors of Src to determine their effect on VEGF expression. Using the Src inhibitor PP2, we demonstrated a decrease in VEGF expression correlating with decreased Src activity. While suggestive of Src regulating VEGF expression, the lack of selectivity of this inhibitor does not allow definitive conclusions with respect to Src regulation of VEGF expression. Therefore, as originally proposed, we have generated clones of

the low-metastatic PC3 cells that ectopically express Src. These clones are required for the completion of all the proposed tasks, and have been a focus of the studies of year 1. Two strategies were used to generate these clones. First, we transfected PC3 cells with a Src expression vector (Src "driven" from a CMV promoter in PCDNAIII, expressing a neomycin-resistance gene) that we have used in the past in other cell types (Windham et al., 2002). G418-resistant colonies were isolated and expanded. Several colonies were obtained with increased Src expression. Some of the PC3-transfectants ectopically expressing Src are shown in Figure 1. Next, as described in the original proposal, these clones were compared to PC3 parental cells for expression of VEGF. To accomplish this portion of the task, cells were grown for 48 hours in complete medium, then changed to medium with 1% fetal bovine serum, supernatant was harvested 24 hours later and VEGF was assessed by ELISA. Results of VEGF expression in some of the clones are shown in Figure 2. The results demonstrate that increased Src directly leads to increased VEGF expression.

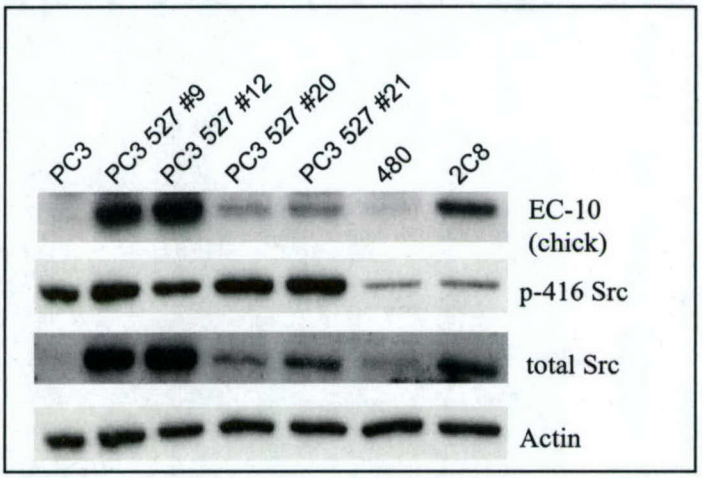


Fig. 1. Generation of PC3 clones expressing activated Src. PC3 cells were transfected, single-cell cloned, and expanded. Immunoblotting was performed with EC 10 to detect the ectopically expressed chicken Src; with an antibody specific for phosphorylated tyr 416 to estimate Src activation, and with Mab 327 for total Src. As controls, SW480 colon carcinoma cells and SW480-2C8, a previously derived clone expressing the activated Src (Windham et al., 2002) were used.

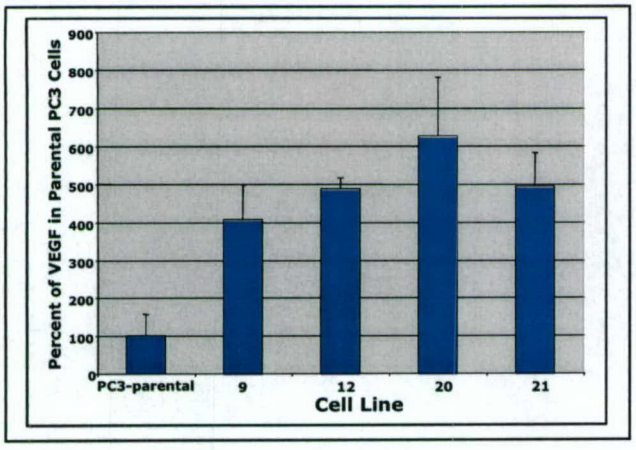


Fig. 2. VEGF expression is increased in Src-transfected PC3 clones. VEGF expression (determined by ELISA from culture supernatant) was increased in all clones expressing activated Src.

As a second approach to increasing Src expression in PC3 cells, we used a tetracycline-inducible vector system. This system has the advantage of direct comparison of Src induced in the same cells, thus avoiding concerns due to clonal variation.

PC-3 cells were transfected with a plasmid that expressed the TET-on protein (Clonetech) and stable clones were selected with G418. PC-3 resistant G418 clones were screened for expression of the tet-protein by transiently transfecting with luciferase plasmid that was induced by the tet-protein in the presence of doxycyclin. Positive clones were then transfected with the secondary plasmid with the Src gene downstream of the tet-binding protein promoter and selected by hygromycin. Resistant clones were checked for Src- expression by treating clones for 48 h in presence of 1.5 μg/ml doxycylin and running western analysis with the EC10 antibody, specific for chicken c-Src. We first designed and selected clones with the activating Y527F mutation, as they would be expected to be more tumorigenic. Several inducible clones were obtained, two of which are shown in Figure 3. As shown in Figure 3, Src is induced to high levels in these clones,

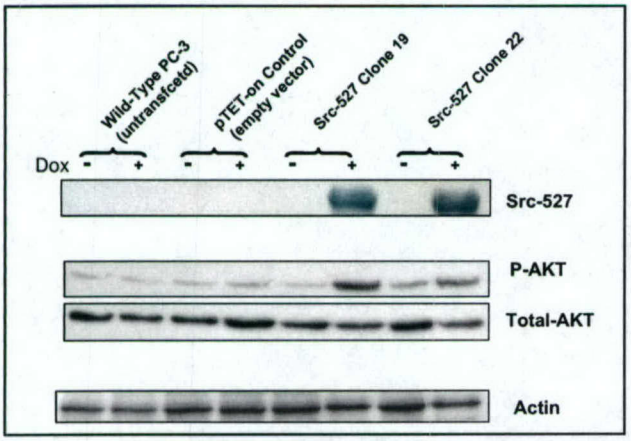
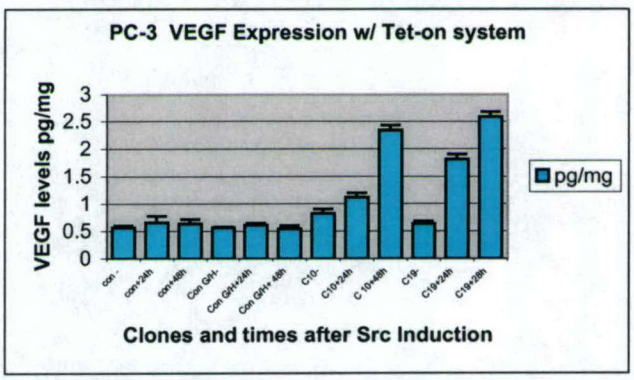


Fig. 3. Expression of Src from a doxycyclin-inducible expression system in PC-3 cells. Shown in the Figure are Src expression in untransfected, pTET-on only (empty vector) controls, and two clones in which Src Y527F is induced. NOTE: EC10 recognized the ectopically expressed chicken Src gene; hence endogenous Src in PC3 cells is not detected. Phosphorylated and total Akt are shown in the middle panels; actin as a loading control is displayed in the bottom panel.

and as expected, Src induction leads to phosphorylation of Akt. The effect of Src induction on VEGF expression was next determined. As shown in Figure 4, control and non-Src induced clones secrete similar amounts of VEGF.

Fig. 4 Expression of VEGF in Src-inducible and control PC3 clones. VEGF in the supernatant was determined by ELISA as described in the proposal. The first three bars represent an untransfected control; the next three bars a PTET-on (empty vector) control. The last sets of three bars indicate VEGF expression from two Src-inducible clones (after indicated times of doxycyclin addition). Induction was measured after 24 and 48 hours.



Upon addition of 1.5 μg/ml doxycylin, VEGF is induced to levels approaching four fold that observed in uninduced and control cells. We have begun tumorigenicity studies on these clones as part of Task 3. The results are described under progress for this task, below. Thus, by multiple approaches we have demonstrated that Src regulates VEGF expression in prostate tumor cells, essentially completing Task 1.

Task 2

Task 2 is to determine the role of c-Src expression in inhibition of apoptosis. This task has commenced as yet, although completion of the task required the generation of the cell lines described above. We anticipate work on this task will commence in year 2.

Task 3

Task 3 is to determine the effect of Src expression on growth and metastasis of human prostate tumor cells in orthotopic mouse models. As indicated in the introduction, there are two mouse models to be employed in these studies: "standard" nude mice and Src-/- nude mice, the latter of which need to be developed as described in the initial proposal. We have begun both components of this task. First, we determined the ability of the tet-inducible PC3/Src clones to grow subcutaneously in nude mice. For these studies, 5 x 10^5 cells were injected subcutaneously into nude mice. Five mice were given standard water; to an identical group of 5 mice, 10mg/ml doxycyclin was added to the drinking water. Each of the mice developed tumors. However, as shown in Figure 5, tumor growth was considerably more rapid in animals receiving doxycyclin. Examination of Src expression in the tumors is in progress. However, the non-"leakiness" of these clones suggest that this strategy offers advantages to selecting stable transfectants shown above; therefore use of the inducible system will become the primary strategy for regulating Src expression for in vivo studies. Thus, we will begin orthotopic injections and metastasis studies in the coming year.

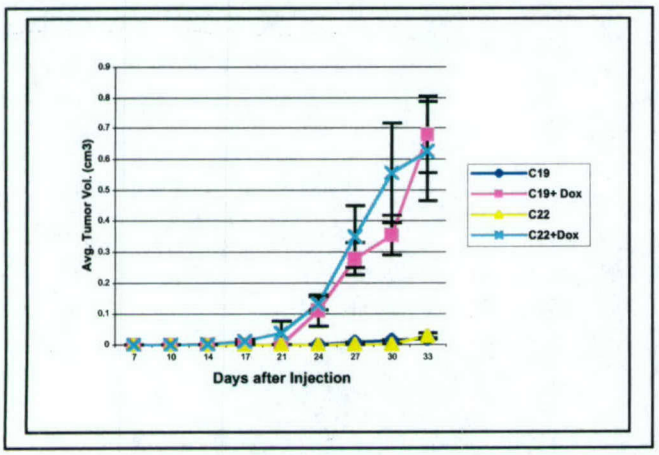


Fig. 5. Growth of tumors from Src-inducible PC-3 sublines. Cells were injected subcutaneously, and mice were given drinking water with or without doxyclin, as indicated in the Figure. Tumor volume was determined as described in the proposal

Generation of Src-/-nu/nu mice

This task has commenced. As described in the proposal, 8 generations of back breeding will be required to obtain Src-/- mice in the desired background. We are currently on our second back breeding. To determine that we can correctly identify the Src-/- phenotype, DNA from tail snips is purified, and Polymerase Chain Reaction (PCR) performed to identify the wild type and mutant allele. An example of the PCR results is shown in Figure 6. As can be seen, both wild type (200bp product) and mutant (450bp product) can be identified in the heterozygous mice; whereas only the wild type allele is identified in homozygous (wt) mice. Thus, we are successfully able to screen for progeny with the correct phenotype. We will continue the breeding in year 2, and expect to be able to perform tumorigenicity studies in the Src-/-nu/nu mice in year 3.

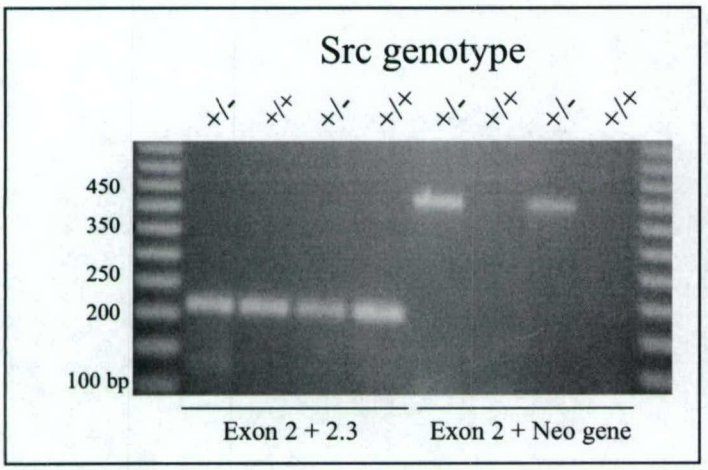


Figure 6. Genotyping of mice from F1 of Src+/- bred to Src+/+nu/nu. DNA is obtained from tail snips and subject to PCR using primers that recognize wild type and interrupted Src alleles. Genotype is shown at the top. As described in the text, wild type alleles generate a 200bp fragment; interrupted (mutant alleles yield a 400bp fragment.

KEY RESEARCH ACCOMPLISHMENTS

- Reduction of c-Met expression reduces VEGF expression in a Src-dependent manner
- Ectopic expression of PTEN/MMAC does not regulate Src activity or VEGF expression
- PC3 human tumor cell subclones were developed that constitutively or inducibly express Src
- Increased Src activity increases VEGF in PC3 cells
- Increased Src in PC3 cells increases tumor growth in nude mice

REPORTABLE OUTCOMES

Regulation of VEGF by Src is a novel result that is reportable, and should result in a publication next year. C-Met regulation of Src and VEGF was reported as follows:

Expression of Vascular Endothelial Growth Factor in Prostate Cancer Cells is Regulated by the non-receptor Protein Tyrosine Kinase, Src. Artime, MC Johnson, M, Parikh, NU and **Gallick, GE** Proc. 95th Annual Meeting of the American Association for Cancer Research, 2004. abst#113.

CONCLUSIONS

We have demonstrated that Src activation leads to increased tumor growth of prostate tumor cells. Tumor growth is associated with increased VEGF expression, suggesting that Src regulates an angiogenic phenotype. Our results therefore suggest that Src activation may be important in progression of prostate cancer, and that inhibition of Src may be of therapeutic benefit. As new Src inhibitors are in clinical trial, these results provide impetus for clinical trials on prostate cancer.

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