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This project is aimed at determining the importance of "S14", a nuclear protein that signals for increased lipid synthesis in normal cells, to breast cancer biology. Our aims are first, *to develop a model of anti-S14 breast cancer therapy in mice*. Intratumoral adenoviral delivery of an S14-antisense gene into human breast cancer cell xenografts caused a significant inhibition of tumor growth. Efforts are now directed at verifying the specificity of this effect using hairpin inhibitory RNA. To this end, we have tested two siRNAs in breast cancer cells in tissue culture, and achieved a major knockdown of S14 mRNA. Second, *to define the stucture of the S14 multimer by X-ray crystallography.* S14 has proven very difficult to crystallize because of folding. We have defined the structure of peptides representing multimerization domains, indicating tetramer formation and the importance of specific residues. Third, *to define the utility of S14 as a clinical marker*. We developed excellent monoclonal antibodies for human S14, and have nearly completed immunohistochemical study of 100 cases (for S14, cyclin D1, and fatty acid synthase) to correlate with clinical markers. The most exciting result is that *S14 is grossly overexpressed in the earliest stage of breast cancer* (DCIS).

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Introduction

This project is aimed at defining the potential of a protein termed "S14" as a therapeutic target in breast cancer. S14 is a small, primarily nuclear protein that signals for increased fatty acid synthesis in normal tissues, such as lactating mammary, liver and adipose, in response to fuel-related hormones and nutrients. Breast cancers are known to have high rates of lipid synthesis, and this promotes their growth and survival. In breast cancer cells, the S14 gene is overexpressed, and this drives their growth. Our aims are to (1) Assess the efficacy of disruption of S14 gene expression in an animal model of breast cancer, (2) Use X-ray crystallographic analysis of purified human S14 to describe the precise geometry of the intermolecular interface in the S14 homotetramer, and (3) Determine S14 expression in an available panel of well-characterized primary human breast cancers, and correlate expression with that of fatty acid synthase (FAS) FAS, cyclin D1, and traditional prognostic factors as well as with disease outcome.

Body

This progress report is organized around the original statement of work, which is reproduced and annotated in this section.

Aim #1: Assess the efficacy and toxicity of disruption of S14 gene expression in an animal model of breast cancer.

- Raise sufficient amounts of recombinant adenoviruses in HEK293 cells, purify and titer the viruses. Months 1-4

This was accomplished, and was facilitated by a new titering procedure using immunocytochemistry for an adenoviral capsid protein.

- Obtain *nu/nu* mice, establish tumor model in pilot study (estimate 15 mice). Months 4-6

This took somewhat longer than expected, as tumors did not form in our initial pilot studies. Injection of a larger number of MCF7 cells solved this problem.

- Inject mice with breast cancer cells, treat with adenoviruses (estimate 30 mice). Months 6-9

This was accomplished, and data regarding the key variable, tumor growth, are shown in **Fig. 1**. A significant reduction in tumor growth was observed in response to the S14-antisense, as opposed to the control (ß-galactosidase) adenoviral treatment. Due to concerns about the specificity of the antisense effect, we are assessing a complementary model using a short hairpin inhibitory RNA (shRNA). We have tested 6 candidate short inhibitory RNAs (siRNAs) in order to identify a candidate(s) shRNA that could be used either in adenovirus or in a mammary-specific transgene. We used fluorescently-tagged siRNAs to assure adequate transfection efficiency in T47D breast cancer cells. As shown in **Fig. 2**, this resulted in a substantial knockdown of S14 mRNA in the cells.

- Perform analyses, analyze data. Months 9-12

Efforts are now proceeding as described above.

- Repeat the above. Months 12-18

Efforts are now proceeding as described above.

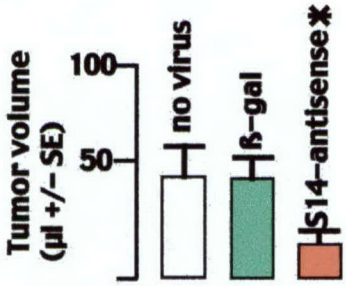

Fig. 1: Effect of adenoviral delivery of S14 antisense mRNA on the growth of MCF7 human breast cancer xenografts in nude mice. Ovariectomized female nude mice with implanted subcutaneous slow-release estrogen pellets (at least 4/group) were injected with 2 x 10^6 MCF7 cells in 50% matrigel into the right and left inguinal mammary fat pads. After 10 d, tumors received 3 sequential treatments, at 6 d intervals, of intratumoral injection of adenovirus harboring either a control gene (ß-gal) or a full-length rat S14 cDNA in the antisense orientation. Tumors were measured with calipers and the volume calculated using a standard formula. Terminal volumes are shown (mean +/- SE, * $p < 0.05$ compared to control or no virus).

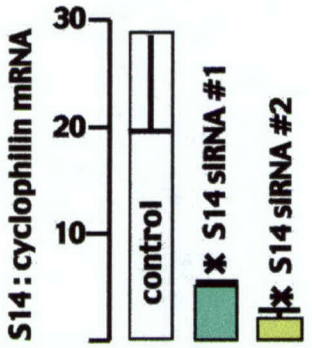

Fig. 2: Identification of effective S14 siRNAs. T47D breast cancer cells were grown to 70% confluence in 6 well plates, and transfected with a scrambled siRNA (negative control) or two candidate fluorescently-tagged S14 siRNAs (4 µg/6 well). Transfection efficiency, assessed by FACS, was 86%. Cells were treated with progestin (10 nM R1881) to induce S14 expression, and RNA was harvested 48 h later for real-time RT-PCR analysis, using primers specific for S14 and cyclophilin, which does not vary. Data are arbitrary units derived from comparison with a standard curve, mean +/- SE (4 wells/group). *$p < 0.05$.

Aim #2: Use X-ray crystallographic analysis of purified human S14 to describe the precise geometry of the intermolecular interface in the S14 homotetramer.

- Subclone the human S14 cDNA into the pROEX-HT vector, express and purify protein. Months 1-6

This was accomplished. The sequenced cDNA was subcloned, expressed in bacteria a a 6 x HIS fusion, and purified on Ni-agaraose, followed by cleavage of the tag with TEV protease and removal of the protease and cleaved tag to yield pure human S14. This material was also used as an immunogen to elicit antibodies used for for Aim 3.

- Assess crystallization conditions, optimize. Months 6-10

This has proven to be difficult. Purified protein produced in our laboratory was used in crystallization screens in Dr. Amy Anderson's laboratory. Analysis using a panel of "standard" crystallization conditions showed the protein to be coagulated but not crystallized. Dr. Anderson has subsequently used > 500 different sets of conditions, to no avail. We have analyzed the bacterially-expressed protein on denaturing and nondenaturing gels, in an attempt to visualize interfering contaminants (of which none were seen), and to assess the folding and multimerization of the protein under nondenaturing conditions. This revealed that 1) the protein does form multimers that migrate consistently with a tetramer, and 2) that there are two forms of the monomer that appear to be folding variants, the "proper" one being the least abundant. We are attempting to maximize production of the desired form by altering the conditions under which the protein is produced in the bacteria by manipulating the temperature and ionic strength of the bacterial growth media. This effort is underway.

Dr. Anderson has undertaken supplementary analyses using 1) computer modeling the carboxyl-terminal multimerization domain to predict key residues for the interaction (**Fig. 3**), and 2) attempts to crystallize peptides corresponding to the interaction domain. The former predicts a key role for tyrosine 138 and lysine 127 in the interaction. We have mutated Tyr138 to alanine, and expressed the protein in bacteria, followed by western analysis. This indicated that the mutation had a profound effect on the physical chemistry of S14, as it rendered the protein totally insoluble in the bacterial pellet (**Fig. 4**).

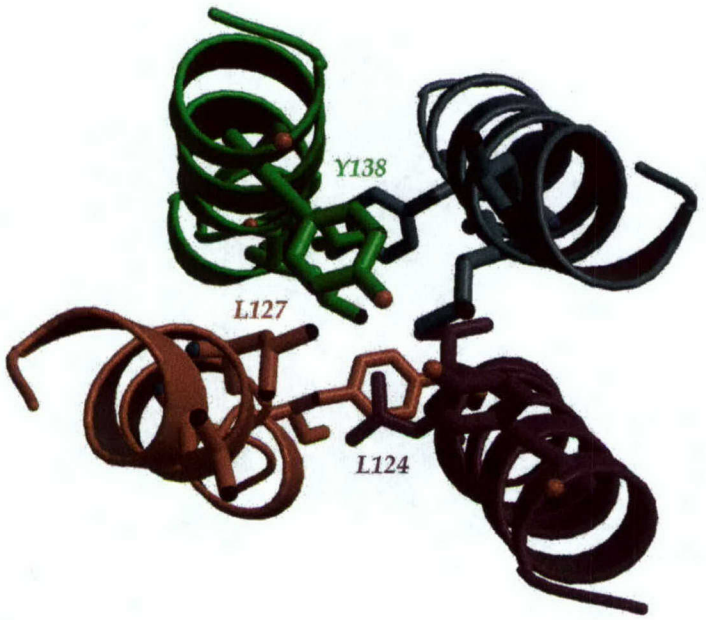

Fig. 3: Computer simulation of interactions between the carboxyl-terminal helical hydrophobic zipper domains of four S14 molecules. Tyrosine 138 and lysine 127 are predicted to be critical for homotetramer formation.

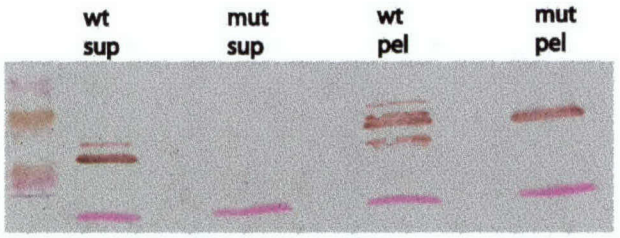

Fig. 4: Western analysis of wild type (wt) and TYR138ALA mutant (mut) S14 in bacteria. Sup denotes soluble supernatant, pel the insoluble pellet. Proteins 25 µg/lane) were analyzed using an antibody specific for human S14. Brown indicates S14 immunoreactivity; pink bands at the bottom of the gel are Pyronin Y tracking dye.

- Obtain X-ray crystallographic structure. Months 10-24

Efforts are directed as described above.

- Validate critical residues in *in vivo* systems (yeast two-hybrid, co-immunoprecipitation), define effects of candidate dominant negative mutations in breast cancer cells. Months 15-36

This awaits crystallization.

- Model drug candidates based on the above information, test in cell culture and mouse model system. Possibly Months 30-36, and beyond

Aim #3: Determine S14 expression in an available panel of well-characterized primary human breast cancers, and correlate expression with that of FAS, cyclin D1, and traditional prognostic factors as well as disease outcome.

- Optimize nonstandard immunohistochemical techniques (ie- those not in general clinical use, including S14, and FAS). Months 6-12

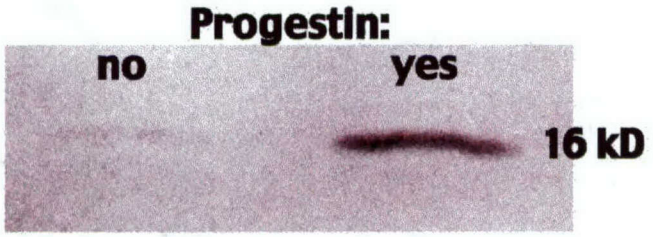

Fig. 5: Detection of S14 protein in T47D breast cancer cells by western blot using monoclonal anti-S14 antibody "#2". Cells grown in charcoal-stripped fetal calf serum were treated with progestin (10 nM R1881) or vehicle for 48 h. Cellular extracts (100 µg protein/lane) were analyzed using crude hybridoma supernatant at 1:1000 dilution.

This has been accomplished. The major issue in this instance was the nonspecific staining seen with our previous polyclonal anti-peptide human S14 antibody preparation. We have worked with Dr. Peter Morganelli, Norris Cotton Cancer Center (Dartmouth Medical School), to develop highly specific monoclonal antibodies for this project. We used the bacterially-expressed human S14 used in Aim #2 as an immunogen in mice for this Aim, and also used it to screen for positive clones by ELISA. We have identified two antibodies that show single bands of the appropriate size on western analysis of human breast cancer cells (results for antibody "#2"

shown in **Fig 5**), and yield excellent results in immunohistochemistry of human breast cancer sections (representative result shown for antibody #2 in **Fig. 6**).

- Cut blocks, perform immunohistochemistry. Months 12-18

This is completed for cyclin D1 and FAS on all 100 blocks. Twenty samples remain to be analyzed for S14.

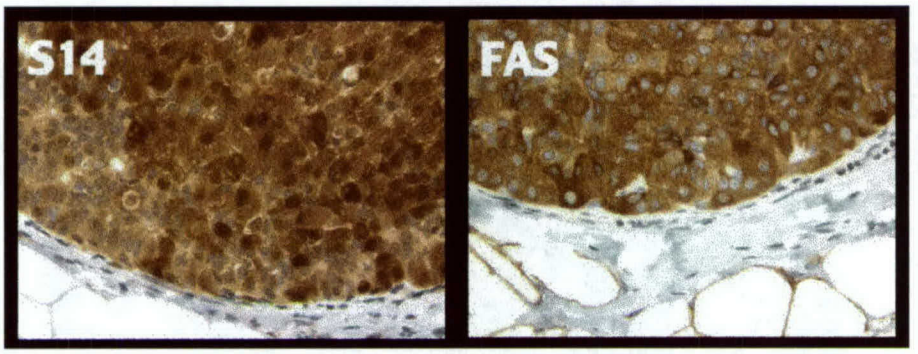

Fig. 6: Examples of immunohistochemical analysis of a ductal carcinoma in situ (D.C.I.S.) case for S14 and fatty acid synthase (FAS) protein expression. Brown pigment indicates immunoreactivity; blue is hematoxylin counterstain. Orginal magnification 40x. S14 shows both nuclear and cytosolic localization. FAS is exclusively cytosolic, as expected. Very high levels of expression of both antigens are seen. Note positive staining for FAS in adipose cells as well. S14 was stained with monoclonal antibody #2, FAS with a commercial polyclonal antibody raised against a synthetic peptide.

- Score samples for marker expression. Months 18-24

This is underway.

- Collate clinical variables. Months 1-24

Data regarding clinical course, histological stage and grade, response to therapy, and expression of standard clinical markers have been collated by Dr. Schwartz.

- Perform statistical analyses. Months 24-30

This awaits completion of the immunostaining.

Key Research Accomplishments

- Preliminary demonstration of efficacy of anti-S14 gene therapy in an animal model.
- Expression of human S14 protein in bacteria.
- Identification of tyrosine 138 as critical for solubility of S14.
- Development of excellent anti-S14 monoclonal antibodies useful in Western and immunohistochemical analyses.
- Demonstration of brisk expression of S14 in the earliest stage of breast cancer (DCIS), to a level equal to that seen in lactating mammary epithelium.

Reportable Outcomes

Thus far, the finding of very high levels of S14 in DCIS is the finding closest to a reportable stage. If the antisense experiments will be able to be reported if the siRNA/shRNA approach confirms the findings.

Conclusions

- S14 antisense gene therapy inhibits breast cancer growth in an in vivo model.
- The amino terminus of S14 forms tetrameric complexes in solution.
- Tyrosine 138 is a key determinant of the physical chemistry of S14, in that mutating it renders the molecule insoluble.
- Highly specific anti-human S14 monoclonal antibodies are useful in the analysis of clinical samples, and show high levels of S14 expression in ductal carcinoma in situ.

References

none

Appendices

none