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14. ABSTRACT

The tasks outlined in the Statement of Work for the first year of research on the action of ADAM9 isoforms in tumor-stromal interactions encompassed the creation of reagents to properly and thoroughly study the problem, as well as preliminary work studying exogenous ADAM9 in a cell-based system. The major developments during this research period were preliminary data showing that the longer form of ADAM9 (ADAM9-L) enhances the migration of breast cancer cells, and that this is dependent on it's metalloprotease activity, and the development of an ADAM9-S(secreted form) antibody, which allows us to detect both isoforms endogenously, allowing for the development of more rigorous models using siRNA and endogenous protein to more thoroughly test our hypothesis.

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ADAM9, Tumor-Stromal Interactions, Breast Cancer, metastasis, migration

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Introduction

The purpose of this research project is to evaluate the molecular mechanisms by which the isoforms of ADAM9 participate in breast cancer progression. We hypothesize that ADAM9 participates in tumor-stromal interactions in breast cancer, acting to enhance tumor progression by mediating HB-EGF shedding and EGFR signaling, and participating in the epithelial-mesenchymal transition through its integrin binding function. ADAM9 isoform expression in the tumor and stroma of human breast cancers will be determined using immunohistochemistry of tissue sample arrays corresponding to different tumor grades and breast carcinoma and stromal cell lines. Molecular analysis of HB-EGF shedding and EGFR signaling will be achieved using mutant constructs of both the membrane-tethered and secreted isoforms of ADAM9 with immunoblotting and functionally evaluated using Matrigel invasion assays. Mutant constructs, immunohistochemistry and immunoblotting will also be used to decipher the molecular mechanism by which ADAM9 participates in the epithelial-mesenchymal transition by binding to integrins expressed pre- and post-EMT. The results of this research will provide us with a new mechanism by which the ADAM9 isoforms present in breast tumors and stroma mediate tumor progression by shedding growth factors and mediating integrin binding. Increased understanding of these mechanisms will lead to new strategies for therapy targeted specifically to advanced stage tumors. This research is also designed to broaden the knowledge, skills, and experience of the Principal Investigator and to provide the foundation for her career in breast cancer research.

Body

The following tasks from the Statement of Work for this project were the focus for the research period from 30 March 2006- 30 March 2007:

Task 1: To characterize the expression of ADAM9 in breast cancer tissues and cell lines representing different stages of the disease (months 1-13).

a. Characterize an ADAM9-S isoform antibody for use in immunoblot and immunohistochemistry (months 1-6).

b. Obtain tissue arrays of human breast cancer specimens and breast cancer and stromal cell lines. Perfect immunohistochemical and RT-PCR techniques and reagents to evaluate the expression of ADAM9 and appropriate controls for tumor and stromal cell types (months 1-2)

c. Characterize the expression of ADAM9-L in the tumor and stromal components of breast cancer specimens on a tissue array using immunohistochemistry and in cell lines using immunoblot and RT-PCR(months 2-7).

d. Characterize the expression of ADAM9-S in the tumor and stromal components of breast cancer specimens on a tissue array using immunohistochemistry and in cell lines using immunoblot and RT-PCR(months 7-13)

Task 2: Determine the role of ADAM9 in HB-EGF shedding and EGFR signaling in breast carcinoma cells (months 7-26)

a. Obtain breast carcinoma cell lines that lack ADAM9 expression as well as express HB-EGF on the cell surface and EGFR. Evaluate these cell lines for invasion upon overexpression of both ADAM9 isoforms (months 7-9).

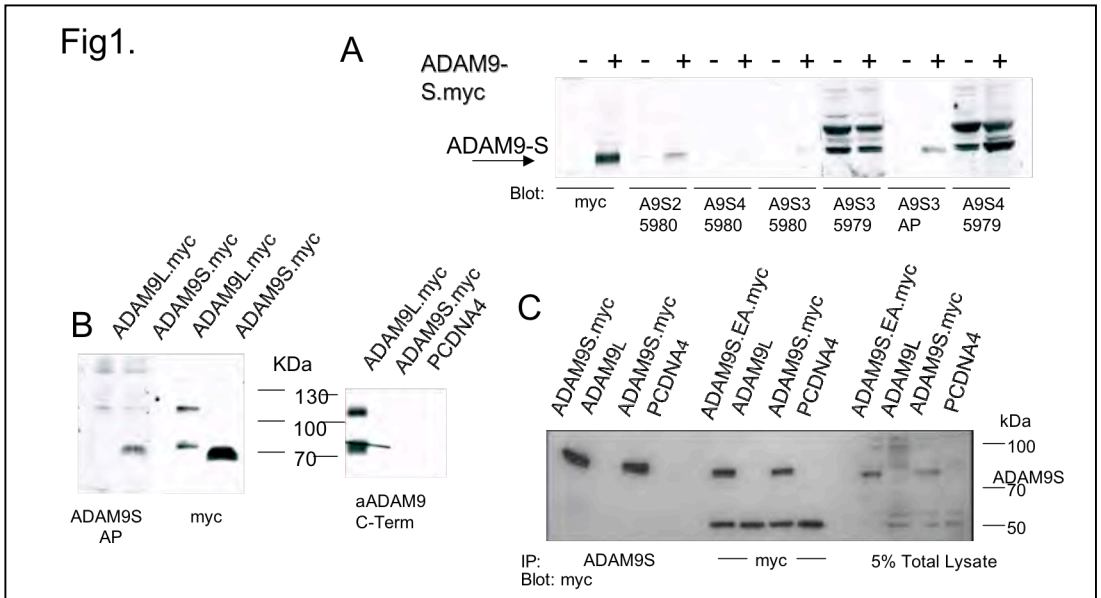
b. Design and evaluate for expression ADAM9-L and -S constructs that contain point mutations and deletions in the metalloprotease, disintegrin, cysteine-rich and, in ADAM9-L, the SH3 domains (months 7-13).

The body of this summary will evaluate the progress made on each of these tasks, problems encountered, and immediate future directions.

Task 1 To characterize the expression of ADAM9 in breast cancer tissues and cell lines representing different stages of the disease.

Progress

Obtaining isoform-specific antibodies to ADAM9 which recognize endogenous proteins

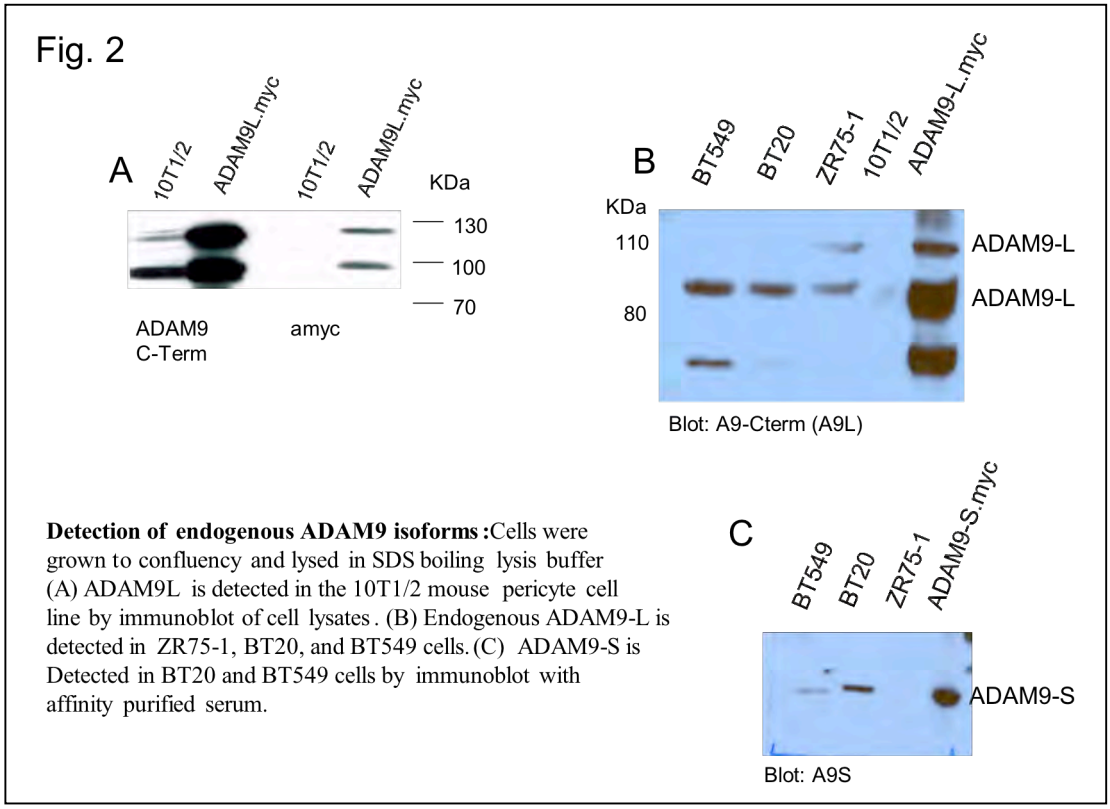


Detection of ADAM9 isoforms using specific antibodies: (A) HEK-293T cell lysate overexpressing PCDNA4 or ADAM9S.myc was blotted with myc, or different antibody bleeds (bleed number delineated by A9S(bleed number) (rabbit#/AP affinity purified)). Rabbit 5980 produced reactive antibody. (B) Cell lysate overexpressing myc-tagged ADAM9-S or L is blotted with ADAM9-S or -L specific antibody to show isoform specificity (C) Overexpressed ADAM9-S.myc is immunoprecipitated using both ADAM9-S and myc antibodies. Proteins

in tissues and cell lines was prioritized in Task 1, as it is required before embarking on cell line and tissue array studies of ADAM9. Task 1A began with the commission of two isoform specific antibodies to the C-Terminal regions of ADAM9-S and ADAM9-L from ProSci Inc. Two rabbits were inoculated with a peptide specific to each construct, and 4 bleeds of serum were obtained from each rabbit, as well as an affinity purified fraction of the third serum bleed. Figure 1 summarizes the results of immunoblotting with each of these bleeds for overexpressing ADAM9-S and ADAM9-L (Fig 1 A, B). The resulting ADAM9-S antibody was further tested to confirm isoform specificity (Fig 1B) and immunoprecipitation (Fig 1C). Our ADAM9-L antibody was not specific, and a commercial C-Terminal ADAM9-L antibody was purchased from BioMol Inc. and tested for specificity (Fig 1B). These initial tests were performed using overexpressed ADAM9.myc constructs to obtain concrete positive controls, so it was imperative to evaluate the detection of endogenous ADAM9 before embarking on tissue arrays and immunohistochemistry. A panel of cell lines (Table 1), primarily breast cancer derived, was selected based on availability and previous literature indicating the expression of ADAM9[1]. Initial results were confounding, as ADAM9-L did not appear to be expressed in cells previously cited in literature (Data Not Shown). However, screening of multiple cell lines unexpectedly revealed ADAM9-L expression in 10T1/2 cells, a mouse pericyte cell line, giving us a positive control for endogenously expressed ADAM9-L (Fig. 2A). As these studies were being performed, Dr. Joe Gray's lab published two papers in Cancer Cell that contribute to both the breast cancer field and my studies. The first identified genomic aberrations linked to breast cancer severity and recurrence, identifying chromosomal regions amplified in more severe disease. ADAM9

Table 1. Original cell lines for proposed research

Cell Line	Properties
SUM159PT	Highly invasive breast carcinoma
MDA-MB-231	Highly invasive breast carcinoma
MCF10A	Immortalized non-tumorigenic breast cell line
MCF-7	Moderately invasive breast carcinoma
SKBR3	Breast carcinoma cell line EGFR+
T47D	Non-invasive breast carcinoma
AU565	Her2+ breast carcinoma
10T1/2	Pericyte cell line, Express ADAM9-L



Detection of endogenous ADAM9 isoforms : Cells were grown to confluency and lysed in SDS boiling lysis buffer (A) ADAM9L is detected in the 10T1/2 mouse pericyte cell line by immunoblot of cell lysates. (B) Endogenous ADAM9-L is detected in ZR75-1, BT20, and BT549 cells. (C) ADAM9-S is Detected in BT20 and BT549 cells by immunoblot with affinity purified serum.

is present in one of these regions, chromosome 8p11-12 [2]. Their second paper examined 54 breast cancer cell lines and identified those that amplified and overexpressed each gene in the amplified chromosomal regions found significant in the previous paper [3]. They identified 13 cell lines in which ADAM9 is amplified or overexpressed, and placed them into 3 groups--luminal, basal A, and basal B, based on phenotype and transcriptional profile based on microarray expression data (Table 2). I purchased three of these cell lines from ATCC (BT20, BT549, ZR75-1), and verified expression of ADAM9-L (Fig 2B). Blotting with ADAM9-S isoform specific antibody also showed that BT549 and BT20 cells also endogenously express ADAM9-S (Fig 2C), the first time this has been shown in a breast cancer cell line.

Table 2. Cell Lines which Overexpress or Amplify ADAM9

Luminal	Basal A	Basal B
CAMA1	BT20	BT549
MDA-MB-134VI	HCC1569	HCC38
SUM185PE	SUM225CWN	
SUM44PE		
SUM52PE		
UACC812		

Current Research and Future Directions

The goal of this task is to develop immunohistochemical tests to determine the presence of ADAM9 in breast cancer tissue arrays, and to ultimately evaluate the isoform distribution in the tumors and stroma of cancers with different clinical profiles. Current research in the scope of this task is dedicated to development of protocols to evaluate the efficacy and specificity of my isoform specific antibodies in fixed-cell

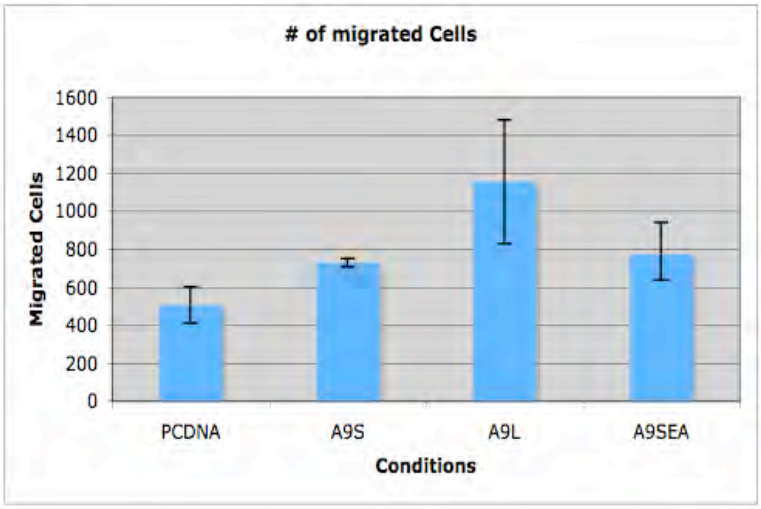
assays such as immunofluorescence and immunohistochemistry. I am currently visualizing endogenous ADAM9-L and ADAM9-S in BT20, BT549 and ZR75-1 cell lines and verifying their subcellular localization. My immediate future work on this task will be to evaluate the antibody in the immunocytochemical process, and then move into tissue array experiments as discussed in this research proposal.

Task2 Determine the role of ADAM9 in HB-EGF shedding and EGFR signaling in breast carcinoma cells

Task 2 depended heavily on accurate detection of expression of overexpressed ADAM9 constructs. Both Tasks 2a and 2b used the SUM159-PT invasive breast cancer cell line to evaluate the role of ADAM9-S and ADAM9-L and functional mutants in the metalloprotease domain in migration assays. We used myc-tagged human ADAM9-S and ADAM9S.EA (metalloprotease deficient) generated in our lab, as well as myc-tagged ADAM9-L, untagged murine ADAM9-L and murine ADAM9L-EA (metalloprotease deficient), obtained from colleagues, in these studies.

The first goal was to recapitulate the data in the literature showing that ADAM9-S promotes breast cancer cell migration [4], and to evaluate differences between ADAM9 isoforms in the ability to enhance migration. An additional goal was to determine the role of the metalloprotease domain in this enhancement. SUM-159PT cells were co-transfected with beta-galactosidase and one of ADAM9S.myc, ADAM9L.myc, and ADAM9S.EA.myc, or vector control at a ratio of 1:5 to ensure that all beta-galactosidase expressing cells are also expressing ADAM9 constructs. One day post-transfection, $1x10^6$ cells were seeded on the upper side of three transwell chambers per condition, and $2x10^5$ cells were seeded to measure transfection efficiency. 3T3 conditioned media was placed in the lower wells of the transwell plate, and the cells were allowed to migrate for 2 hours before being stained with X-gal solution to evaluate beta-galactosidase activity. Blue cells were counted to determine transfection efficiency of each condition, and the number of cells migrated. These results were normalized to the transfection efficiency. $1x10^7$ cells in each condition were lysed and immunoblotted for confirmation of protein expression. Figure 3 illustrates the original results of these studies on ADAM9S.myc, ADAM9L.myc, and ADAM9S.EA.myc. We can see an increase in cell migration upon transfection with ADAM9L.myc, but not with

Fig. 3



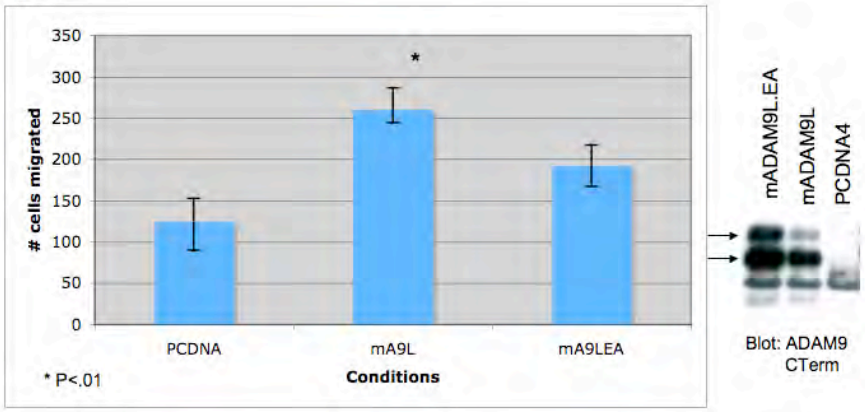
The role of ADAM9 isoforms in the migration of breast cancer cells: SUM159-PT breast cancer cells were transfected with PCDNA vector, ADAM9-S, ADAM9-L, or metalloprotease-deficient ADAM9S.EA plasmids and put through a transwell migration assay as described in the text. The blot represents $1x10^6$ cells lysed and blotted with myc to ensure protein expression

ADAM9S.myc, or ADAM9S.EA.myc. The increase in migration of SUM159-PT cells with the overexpression of ADAM9-L is a new result. The ADAM9-S result fails to support the data obtained in previous studies[4], however, in those experiments, recombinant ADAM9-S is added to cells prior to the assay being performed. It is not clear if the ADAM9-S being produced by the transfected cells would reach the same levels as the exogenous protein, given that ADAM9-S is secreted and the cells are washed before being subjected to the migration assay. The original assays were also performed in an invasion format, using matrigel-coated transwells as a model for invasion through the extracellular matrix.

The preliminary data showing an increase in migration by ADAM9L overexpressing cells was followed up with another migration assay using murine ADAM9L constructs. Vector control, ADAM9L, and the ADAM9L.EA were transfected and the assay carried out in the same manner as above. Transfection of ADAM9L significantly increased the migration of SUM159-PT cells as determined using an ANOVA significance test, and this increase in migration was not seen in cells transfected with the ADAM9L.EA

metalloprotease deficient mutant, indicating a function for the metalloprotease domain in this phenotype (Fig.4).

Fig. 4



Mouse ADAM9-L increases the migration of breast cancer cells through metalloprotease activity: SUM159-PT cells were transfected with PCDNAvector control, murine ADAM9L, or the mA9LEA murine ADAM9L-metalloprotease-deficient mutant. A migration assay was performed as described in the text. mA9L transfected cells migrated significantly faster than the other conditions represented by an ANOVA p value of <.01 The blot is to ensure protein expression, ADAM9-L is marked by arrows.

The availability of breast cancer cell lines that overexpress ADAM9 has moved this project to the next level of productivity. The following current research section describes experiments that will be conducted with these cells, which will complete Task 2.

Current Research and Future Directions

The use of cell lines that endogenously express ADAM9 will change the method, but not the nature of this task. Current research is focused on development of lentiviral siRNA directed to ADAM9, which will allow for the comparison of ADAM9 overexpressing and silenced cells in multiple assays, including migration and invasion. The involvement of ADAM9 in multiple facets of tumor formation and progression may be tested using this method, and my initial results using ADAM9 overexpression validated in a more endogenous setting. The following outline represents the current focus of my research in this area:

1. Knock-down of ADAM9 in endogenously expressing cell lines
2. Observe cells with silenced ADAM9
    1. Phenotype, proliferation, survival
        1. An overt change in proliferation or survival of cells deficient in ADAM9 will lead us to focus on possible ADAM9 substrates such as HB-EGF and EGFR signaling as a mechanism for the function of ADAM9 in tumorigenicity
    2. Migration and invasion assays

1. We expect to see a decrease in migration upon silencing of ADAM9, the opposite of overexpressing ADAM9 in cells. This finding will result in evaluation of ADAM9 domains by reconstituting cells deficient in ADAM9 with proteins that are deficient in the metalloprotease and disintegrin-binding domains.
    1. The establishment of the functionally important domain of ADAM9 in tumor cell migration/invasion will lead us to focus on ADAM9 substrates which may be cleaved or bound by ADAM9 to determine the pathways by which ADAM9 functions.

Key Research Accomplishments

- ♣ Development and characterization of an isoform-specific ADAM9-S antibody
- ♣ Identification of endogenous ADAM9-S in breast cancer cell lines
- ♣ Identification of endogenous ADAM9-L in breast cancer cell lines
- ♣ ADAM9-L promotes the migration of SUM159-PT cells toward 3T3-conditioned media.
- ♣ The metalloprotease domain of ADAM9-L plays a role in the enhancement of cell migration

Reportable Outcomes

- ♣ Development of ADAM9-S isoform specific antibody (should this really be here?)

Conclusion This first annual summary encompasses work done on Tasks 1 and 2 of the Statement of Work for this project. The development of the ADAM9-S isoform specific antibody represents a critical step in delineating the importance of the membrane-bound and secreted isoforms of ADAM9. This antibody has directly lead to the discovery of the ADAM9-S isoform endogenously expressed in breast cancer cell lines. This furthers our hypothesis that ADAM9 acts both on the tumor cell itself and in the surrounding stroma to enhance breast cancer cell migration and invasion, and future work with this antibody will determine expression in actual breast cancer specimens, definitively placing ADAM9 in the breast cancer microenvironment. The migration studies using ADAM9-L show that it enhances migration in breast cancer cells, and that the metalloprotease domain plays a role in this phenotype. This supports the

hypothesis that the ability of ADAM9 to cleave substrates is a functionally important, and supports our work on identifying ADAM9 substrates, which contribute to migration and invasion, beginning with EGF. The availability of breast cancer cell lines that overexpress ADAM9 allows us to use both overexpression and silencing as tools to tease out the function of ADAM9 in cells, and to confirm the function of ADAM9 in multiple cell types. The identification of ADAM9 in a genomic amplification region that contributes to decreased survival and increased recurrence in breast cancer and the recognition that the structure of ADAM9 make it a good candidate for drug therapy [2], make this work critical, not only for confirmation that removing ADAM9 signaling will enhance breast cancer treatment, but also for identifying possible problems and side-effects which may arise with drug therapy.

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