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Award Number: W81XWH-07-1-0461

TITLE: Genetic Dissection of the Role of Heparan Sulfate in Mammary Tumor Progression

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REPORT DATE: June 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| 30-06-2008 | Annual | 1 JUN 2007 - 31 MAY 2008 |

**4. TITLE AND SUBTITLE**

Genetic Dissection of the Role of Heparan Sulfate in Mammary Tumor Progression

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-07-1-0461

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Burnham Institute
La Jolla, CA 92037

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

Heparan sulfate (HS) binds growth factors, protein-degrading enzymes, and other bioactive proteins, and to regulate their activities. Many of these proteins have strong implications in human breast cancer. There is also evidence that cellular HS production itself exerts strong influences on tumorigenesis, exemplified by the fact that mutations of Ext1, the gene encoding an HS synthesizing enzyme, cause multiple bone tumors. Furthermore, the level of HS degrading activity correlates with the aggressiveness of the tumor. Despite these long-standing observations, much less is known about the mechanisms by which HS influences the malignant behavior of tumors in vivo. Also important is the fact that HS is produced not only by tumor cells themselves but also by stromal cells that constitute the tumor microenvironment. This project will conduct cohort study using genetic mouse models to address these key questions. The first year of this project was dedicated mainly to establish, expand, and intercross mouse models for generating experimental cohorts. We have also characterized the gene recombination pattern induced by the FSP1-Cre transgenic mouse, which will be used to manipulate the expression of HS in tumor stromal cells.

**15. SUBJECT TERMS**
Heparan sulfate; Ext1; Tumorigenesis; Tumor microenvironment; Conditional knockout.

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 11 | 19b. TELEPHONE NUMBER *(include area code)* |

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## Introduction

Heparan sulfate is a linear polysaccharide composed of repeating N-acetylglucosamine and glucuronic acid residues. The anticoagulant heparin is a specialized form of heparan sulfate. Heparan sulfate chains are covalently attached to various core proteins to form heparan sulfate proteoglycans (HSPGs). HSPGs exist mainly as cell surface and extracellular matrix molecules and are functionally involved in various biological processes, including growth factor signaling, regulation of morphogen gradient, cell adhesion, lipoprotein metabolism, and modulation of proteinase activities (1). Considering its interactions with a number of growth factors/cytokines, it is likely that heparan sulfate plays an important role in cancer development and progression. For instance, two signaling molecules that have strong implications in human breast cancer, namely Wnt1 and neuregulin (the Ig-domain containing isoforms), bind and functionally modulated by heparan sulfate. Factors known to affect invasion, metastasis, and tumor angiogenesis, such as matrix metalloproteinases, VEGF, and endostatin, also interact with heparan sulfate. Despite this wealth of data, our understanding of the mechanisms by which heparan sulfate influences tumor cell behavior *in vivo* is still fragmentary. One of the important unknowns is what is the overall physiological effect of heparan sulfate on tumor development and progression. Further compounding the issue is that heparan sulfate is produced not only by tumor cells themselves but also by stromal cells within tumors. The field needs advanced animal models that not only closely mimic clinical cancers but also allow precise dissection of heparan sulfate function in different cell types. The key glycosyltransferase for the biosynthesis of heparan sulfate is the glycosyltransferase called Ext1. Ext1 catalyzes the polymerization of N-acetylglucosamine and glucuronic acid residues (2, 3). Genetic and biochemical studies have established that Ext1 is absolutely essential for heparan sulfate biosynthesis (4). These properties make the *Ext1* gene as an excellent target for genetic disruption of heparan sulfate synthesis. This allows direct interpretation of the causal relationship between heparan sulfate and the resultant phenotype. Our primary objective is to obtain direct information regarding the role of heparan sulfate in breast cancer development and progression in the context of *de novo* mammary tumorigenesis models. An ancillary objective is to determine whether tumor cell-expressed heparan sulfate and stromal cell-expressed heparan sulfate exert distinct effects on the behavior of mammary tumors. We hypothesize that they have different effects on the growth and progression of mammary tumors. Through this project, we will define the role of heparan sulfate in breast cancer under the condition that mimics human breast cancer than ever before, thereby advancing our understanding of the role of heparan sulfate in breast cancer to the next level.

## Body

### Task 1. Acquisition of animal experiment approval.

Necessary approval for animal experiments was obtained on schedule.

### Task. 2. Generation of animal cohorts for studies in Aim 1 (the role of tumor cell autonomous heparan sulfate in mammary tumor development and progression).

The original plan calls for the breeding of experimental animals for the *MMTV-Cre/KFS2MT6*-based tumorigenesis study during the first two years of the project. This part of the project has experienced a problem of low fertility of *KFS2MT6* transgenic mice. We obtained *KFS2MT6* transgenic mice (5) from Dr. Robert Oshima soon after the initiation of funding, and first attempted to expand the colony for the preparation of systematic crossbreeding with $Ext1^{flox}$ (6) and MMTV-Cre mice. Unexpectedly, mating of $KFS2MT6^{+}$ mice with wild-type ($KFS2MT6^{-}$) littermates exhibited low frequency of successful pregnancy. We also backcrossed $KFS2MT6^{+}$ mice with unrelated, wild-type C57BL/6 mice with a similar result. We do not know what is the exact reason for this low efficiency of breeding. The *KFS2MT6* transgenic mice obtained were young (~4 weeks old) and should be appropriate for breeding. During the same period, other mouse lines, including *FSP1-Cre* which is also used in this project, showed normal levels of breeding. It may be an intrinsic property of the line. As a possible remedy, we have obtained additional breeding pairs of *KFS2MT6* transgenic mice to expand the basis for breeding.

As a result of this problem, the progress of this part of the project may experience a delay in subsequent years. Meanwhile, we have front loaded the breeding for Aim 2 to compensate the potential delay in Aim 1.

### Task 3. Analysis of mammary tumors in Aim 1.

Because this part of the project is proposed to be conducted in the second and third years, it is not applicable to this report.

### Task 4. Expression profiling of genes between *Ext1* null and control tumors.

Because this part of the project is proposed to be conducted in the second and third years, it is not applicable to this report.

### Task 5. Generation of animal cohorts for studies in Aim 2 (the role of stromally produced heparan sulfate in mammary tumor development and progression).

The original plan calls for this part of the project to be conducted after Aim 1. However, to compensate the anticipated delay in Aim 1, we accelerated this part of the project as follows.

*FSP1-Cre* transgenic mice (7) were obtained from Dr. Eric Neilson of Vanderbilt

University. The mice had white coat color, indicating that they still retain FVB background. Therefore, after conducting mandatory rederivation of the line, we proceeded to backcrossing to the C57BL/6 genetic background, in which all experiments are to be performed. Backcrossing for five generations has been completed ahead of the schedule, and intercrossing to produce experimental cohorts has recently been initiated. Unlike *KFS2MT6* transgenic mice, we have not experienced any difficulties in breeding, although the average size of their litter has been slightly smaller than expected.

To characterize the recombination pattern driven by the *FSP1-Cre* transgene, we have crossed *FSP1-Cre* mice with *Rosa26R* Cre reporter mice (8). One of the questions we wanted to examine with this validation study is how *FSP1-Cre*–mediated recombination occurs in developing embryos. As we discussed in the original application, a concern surrounding this study is that *FSP1-Cre*–mediated recombination may occur extensively in embryonic fibroblasts. Such early recombination could result in severe developmental defects or even embryonic lethality. Thus, we crossed *Rosa26R* reporter mice and *FSP1-Cre* mice, and compound mutant embryos were examined for the expression of β-galactosidase. As shown in Fig. 1, recombination was detected in E12.5 embryos, but was highly restricted to the liver. Moreover, horizontal sections through the trunk demonstrated that recombination was restricted to the surface of the liver. The parenchyma of the liver was not stained for β-galactosidase. Thus *FSP1-Cre* does not induce gene recombination globally in embryonic fibroblasts, and it is rather unlikely that *FSP1-Cre* causes unwanted developmental defects that preclude the proposed experiments, although this needs to be further confirmed by examining the viability of *FSP1-Cre;Ext1*$^{flox/flox}$ conditional mutants. In next litters, we will examine postnatal *FSP1-Cre;Rosa26R* mice to verify the recombination in fibroblasts in the mammary glands.

As discussed in the original application, if *FSP1-Cre*–mediated ablation of *Ext1* results in embryonic or early postnatal lethality, or severe developmental defects in the mammary glands, we will need to employ an alternative approach based on an *ex vivo* grafting model as outlined in the original application. However, considering the result of the recombination pattern analysis, we are optimistic that the proposed *in vivo* study can be conducted as planned.

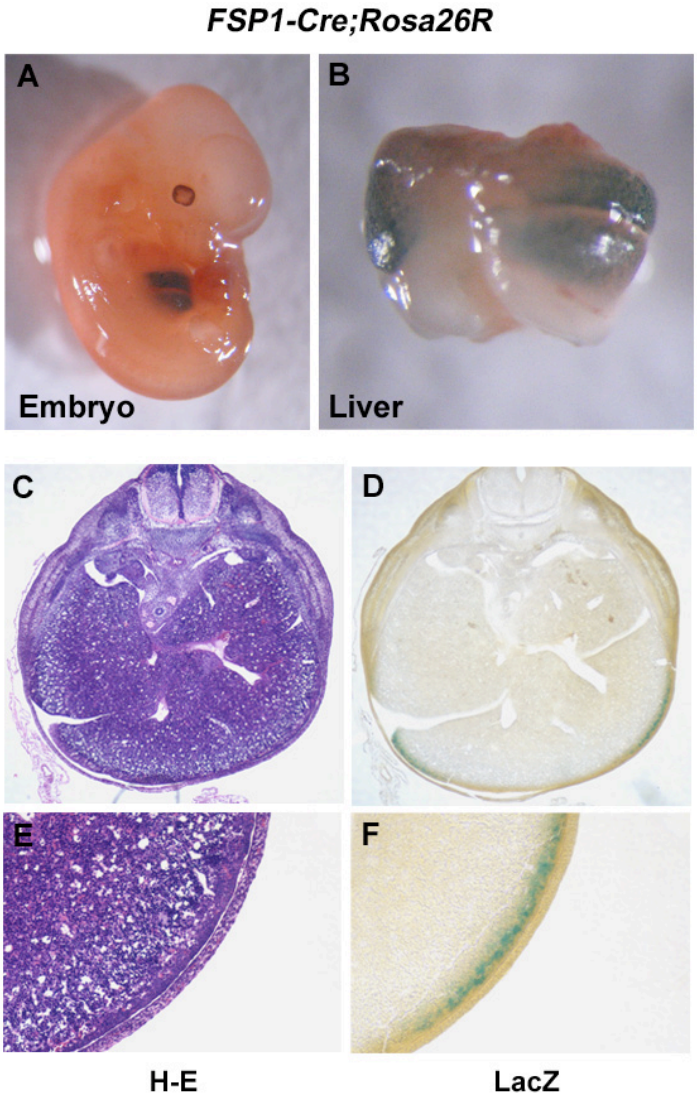


**Figure 1. The pattern of gene recombination induced by the *FSP1-Cre* transgene.** An *FSP1-Cre;Rosa26R* embryo at E12.5 was stained for the β-galactosidase activity with X-gal. (**A**) X-gal staining is detected only in the liver. (**B**) The liver dissected from the embryo shown in **A**. Note that X-gal staining is observed in only part of the liver surface. (**C–F**) Horizontal sections of an E12.5 embryo stained by hematoxylin-eosin (**C,E**) or X-gal (**D,F**). X-gal staining is observed on the liver surface. No staining is observed in the liver parenchyma.

## Key Research Accomplishments

*Establishing colonies of necessary mouse models*

- Obtained *KFS2MT6* transgenic mice.
- Obtained and rederived *FSP1-Cre* mice.
- Changed the genetic background of *FSP1-Cre* mice to the C57BL/6 background by backcrossing for 5 generations.
- Established genotyping protocols for the detection of the *FSP1-Cre* transgene.
- Established genotyping protocols for the detection of the *KFS2MT6* transgene.

*Breeding of experimental cohorts*

- Expanded the colony of *FSP1-Cre* mice in the C57BL/6 background.
- Initiated crossing between *FSP1-Cre* and $Ext1^{flox}$ mice.

*Characterization of FSP1-Cre–mediated gene recombination patterns*

- Bred *FSP1-Cre;Rosa26R* mice by crossing *FSP1-Cre* and *Rosa26R* reporter mice.
- Performed the analysis of gene recombination in *FSP1-Cre;Rosa26R* embryos by β-galactosidase assays.
- Analyzed the pattern of β-galactosidase staining in sections.

*Development of analytic methods*

- Developed an antigen retrieval protocol based on microwave irradiation for the sensitive detection of heparan sulfate in tissue sections.

## Reportable Outcomes

- Abstract for DOD Era of Hope 2008 Meeting (enclosed in the Appendix)

  Matsumoto, K., and Yamaguchi, Y. "Genetic dissection of the role of heparan sulfate in mammary tumor progression"

- Presentations

  Yamaguchi, Y. "Heparan sulfate and cancer" Tumor Microenvironment Program Meeting, Burnham Institute for Medical Research (February, 2008)

## Conclusions

This is the first progress report of this IDEA award. The goal of the project is to obtain physiological evidence for the role of heparan sulfate in mammary tumor development and progression using conditional knockout mouse models. Because of the complexity of the breeding schemes necessary for producing triple compound mutant mice and their control counterparts, the large portion of the grant period is to be spent for crossbreeding of mice. Actual analyses and experiments are to be conducted toward the latter part of the funding period.

The first year has been spent mostly for setting up colonies of necessary mouse models and initial steps of crossbreeding to produce various compound mutant mice. Establishment and expansion of the colony of *FSP1-Cre* transgenic mice, the key model for Aim 2, have been going well and on schedule. To compensate the potential delay in Aim 1, we have front loaded breeding of compound mutants for Aim 2. We have also performed the analysis of *FSP1-Cre*–mediated spatial recombination patterns during embryonic development. *FSP1-Cre* does not induce extensive gene recombination in embryonic fibroblasts, which makes the possibility of embryonic lethality of *FSP1-Cre;Ext1*$^{flox/flox}$ mice less likely. Overall, the progress in Aim 2 is ahead of schedule.

On the other hand, we have experienced the problem of inefficient breeding of *KFS2MT6* transgenic mice, which would cause a delay in the progress of Aim 1. Breeding performance of laboratory mice can be affected by many factors (9) and it is often difficult to identify specific reasons. To remedy this problem, we will increase the number of breeding pairs and check and optimize housing conditions. We are confident that, although the drawback may incur some delay, we will be able to conduct the mouse cohort study as planned. Should this problem persist during the second year, we will turn to the alternative *ex vivo* model as outlined in the original application.

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# View Paper

## GENETIC DISSECTION OF THE ROLE OF HEPARAN SULFATE IN MAMMARY TUMOR PROGRESSION

BC060176

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*Burnham Institute*

**Background and Objectives:** Heparan sulfate (HS) exists mainly as cell surface and extracellular matrix molecules and are functionally involved in various biological processes. Considering its interactions with a number of growth factors/cytokines, it is likely that HS plays an important role in cancer development and progression. Two signaling molecules that have strong implications in human breast cancer, namely Wnt1 and neuregulin (the Ig-domain containing isoforms), bind and functionally modulated by HS. Factors known to affect invasion, metastasis, and tumor angiogenesis, such as matrix metalloproteinases, VEGF, and endostatin, also interact with HS. Despite this wealth of data, our understanding of the mechanisms by which HS influences tumor cell behavior in vivo is still fragmentary. One of the important unknownswhat is the overall physiological effect of HS on tumor development and progression. Further compounding the issue is that HS is produced not only by tumor cells themselves but also by stromal cells within tumors. The field needs advanced animal models that not only closely mimic clinical cancers but also allow precise dissection of HS function in different cell types.

**Methodologies:** Our experimental tool is the conditional Ext1 allele. The key glycosyltransferase for the HS biosynthetic process is the GlcNAc/GlcA copolymerase encoded by the Ext1 gene. Genetic and biochemical studies have established that EXT1 is absolutely essential for HS biosynthesis. We have created loxP-modified Ext1 allele, from which conditional Ext1 knockout mice can be generated by crossing with Cre transgenic mice targeted to various tissue and cell types. In this project, conditional ablation of the Ext1 gene will be combined with polyoma middle T antigen (PyMT)-dependent de novo mammary tumorigenesis models. We will produce MMTV-Cre;KFS2MT6;Ext1$^{flox/flox}$ animals (KFS2MT6 is a PyMT transgene that is activated by Cre-mediated excision of the STOP cassette). MMTV-Cre will activate PyMT expression and disrupt Ext1 concurrently in the mammary epithelium. Therefore, this system will allow us to examine specifically the role of tumor cell autonomous HS. We will also examine PyMT-induced mammary tumorigenesis in the HS-deficient stromal environment. For this, Ext1 will be disrupted specifically in stromal fibroblasts, without interfering HS synthesis in tumor cells, using the FSP1-Cre transgene. This experiment will allow us to determine the role of stromal cell-derived HS in tumor progression. In both experiments, mammary tumor progression will be analyzed in terms of growth, angiogenesis, invasion, and metastasis. Microarray analysis will be performed on tumor RNAs to gain insight into the difference in intracellular signaling between Ext1 null and control backgrounds.

**Results to Date:** We have completed backcrossing all necessary lines to C57BL/6 and are currently breeding experimental and control groups for the tumor analyses.

**Conclusions:** Considering its clear implication in tumor growth and progression, HS is an emerging therapeutic target in breast cancer. Yet our current concept on the role of HS in tumor development is derived mostly from the observations obtained from non-physiological experimental models. This project would have a direct impact on this issue by defining the physiological role of HS in mammary tumor development and progression.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0461.