AD_____

Award Number: W81XWH-06-1-0688

TITLE: Assessment of GPR30, a Seven Transmembrane-spanning Estrogen Receptor, as an Oncogene

PRINCIPAL INVESTIGATOR: Edward Joseph Filardo, Ph.D.

CONTRACTING ORGANIZATION: Rhode Island Hospital
Providence, RI 02903

REPORT DATE: October 2008

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
17-10-2008	Annual	18 Sep 2007-17 Sep 2008

4. TITLE AND SUBTITLE
Assessment of GPR30, a Seven Transmembrane-spanning Estrogen Receptor, as an Oncogene
*

5a. CONTRACT NUMBER
BC052936

5b. GRANT NUMBER
W81XWH-06-1-0688

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Edward Joseph Filardo, Ph.D.

Email: edward_filardo@brown.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Rhode Island Hospital, Providence RI 02903

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
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 Expression of the seven transmembrane-spanning receptor (7TMR), GPR30, in primary human breast tumors is positively associated with several tumor progression variables including extra mammary metastases (Filardo et al, 2006). Altered expression of 7TMRs is linked with a spectrum of disease phenotypes, including cancer, raising the possibility that GPR30 may function as an oncogene. To test this hypothesis, two lines of transgenic mice (T6-1A and T6-2E) were engineered with stably integrated hemagglutinin-tagged (HA-GPR30) transgenes under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter. Endogenous GPR30 protein was readily detected in mammary glands harvested from control and transgenic mice using peptide antibodies. However, breast tissue from nulliparous progeny mice expressed no detectable GPR30 transgene protein or mRNA. MMTV-HA-GPR30 mice exhibited normal reproductive behavior, lactational competence and no overt signs of cancer or premaligancy. Increased transgene expression or abnormal mammary gland growth was also not evidenced in multiparous mice. A second no cost extension was requested to construct mice with an HA-GPR30 transgene regulated by the whey acidic protein(WAP) promoter.

15. SUBJECT TERMS
membrane receptor, estrogen, transgenic mice

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

Table of Contents

Introduction.

Breast tumor growth and survival is strongly influenced by estrogen and decisions regarding appropriate adjuvant therapy for patients with breast cancer are largely determined by the measurement of known estrogen receptors (ERs) in primary tumor biopsy specimens. However, it has long been suspected that receptors other than the known estrogen receptors may promote estrogen action. Recent findings by our lab (1-4), and others (5-8), has shown that the seven transmembrane receptor (7TMR), GPR30, promotes specific estrogen binding and biochemical signaling and in addition is linked to tumor progression in man. To further address the role of GPR30 in experimental breast tumor biology, we have proposed to generate transgenic mice capable of overexpressing wild type or active GPR30 using mammary gland specific promoters.

Body.

Work conducted during no cost extension.

The one-year term of this Concept award expired on October 31, 2007. The overall goal of the concept award was to generate transgenic mice that overexpressed wild-type or active GPR30 for the purpose of assessing the biological role of this newly appreciated membrane estrogen receptor in breast cancer. We successfully generated two founder mice (T6-1A and T6-2E) that contained a stably integrated wild-type hemagluttinin (HA)- tagged GPR30 transgene during that time frame and were unable to identify an active GPR30 allele (see Nov 1, 2006- Oct 31, 2007 progress report). Accordingly, we requested and were granted, a no-cost extension to further evaluate the expression of the transgene in these mice and to determine if there was an association with the development of mammary adenocarcinoma.

The work performed during the no cost extension was limited to Task 1 of the original *Statement of Work*. No efforts were made to pursue the development of an active GPR30 allele as remaining monies did not allow. No salary support was drawn during the no-cost extension. Monies budgeted for the generation of GPR30 CAM mice were used, in part, to conduct breeding experiments that were designed to assess MMTV-HA-GPR30 transgene expression and potential malignancy in the mammary glands of nulliparous, parous and multiparous mice. The results of these experiments are listed below.

Salaries were covered entirely by Departmental Funds from Medicine at Rhode Island Hospital.

Task 1. To evaluate the impact of hyperexpressed wild-type GPR30 on mammary duct branching and predisposition for the development of invasive breast cancer.

Expression of MMTV-HA-GPR30 in T6-1A and T6-2E transgenic mouse strains.

Two lines of mice (T6-1A and T6-2E) harboring stably integrated MMTV-HA-GPR30 transgenes were evaluated for transgene expression. The mouse mammary tumor virus (MMTV) promoter has been used extensively to conditionally express transgenes in the mammary gland (*reviewed* in 9,10). Some variation in

expression has been reported based upon the content of the transgene (*cis* effect) and parity (*trans* effect). To evaluate the expression of the HA-GPR30 in each of these transgenic lines and the potential influence of pregnancy on transgene expression, we generated transgene positive female progeny and then evaluated the expression of HA-GPR30 in nonporous, parous and multiparous mice.

Despite the fact that the transgene was stably inherited, the MMTV-HA-GPR30 transgene protein was not detected in either lineage and was not influenced by the pregnancy status of the mice (**figure 1**). The HA-GPR30 gene product was readily detected when expressed under a CMV promoter in HEK-293 cells suggesting that the inability to express the MMTV HA-GPR30 transgene in mice was related to the promoter (**figure 2**). No indications of preneoplasia or malignancy were observed in any of the transgene positive mice. However, due to the fact that we did not measure transgene expression, we are unable to conclude whether GPR30 is involved in spontaneous mammary adenocarcinoma.

Key Research Elements.

None.

Reportable Outcomes.

None.

Conclusions.

Despite the fact that the transgene was stably inherited in two lines of MMTV-HA-GPR30 mice, the MMTV-HA-GPR30 transgene protein was not detected in either lineage and was not influenced by the pregnancy status of the mice. The HA-GPR30 gene product was readily detected when expressed under a CMV promoter in HEK-293 cells suggesting that the inability to express the MMTV HA-GPR30 transgene in mice was related to the promoter. We have previously shown in work supported internally here at Rhode Island Hospital that WAP-regulated carboxyl truncated HA-GPR30 protein is efficiently expressed in the mammary glands of mice (**figure 3**). We originally proposed to employ a WAP expression vector in our original *Statement of Work* but decided against this based upon the fact that while transgene expression was extremely abundant (as assessed by immunohistochemistry with HA antibodies), the percentage of mammary ducts positive for the transgene varied among litter mates. However, this difference may be overlooked if any positive results are generated demonstrating an association between GPR30 and the development of spontaneous mammary adenocarcinoma. This work is worth pursing since we (4), and others (8), have shown a strong association between GPR30 expression and malignancy in breast and uterine neoplasia in man.

Since, we have clearly shown that the MMTV-HA-GPR30 transgene was not expressed in these mice and therefore cannot make any conclusions about the role of GPR30 in spontaneous murine mammary adenocarcinoma, a second no cost extension was requested to engineer mice that express HA-GPR30 under the influence of a WAP promoter. No additional monies are requested for this work, and all salaries will be supported by internal Funds. This work is significant in that these animals may represent potential interesting models for studying the role of this previously unappreciated estrogen receptor in breast cancer biology.

References.

1. Filardo EJ, Quinn JA, Bland KI and AR Frackelton Jr: 2000. Estrogen-induced activation of Erk-1 and Erk-2 requires the G-protein-coupled receptor homologue, GPR30, and occurs via transactivation of the EGF receptor through release of HB-EGF. *Mol Endocrinol.* 14: 1649-1660.

2. Filardo EJ, Quinn JA, Frackelton AR Jr, Bland KI. 2002. Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endocrinol.* 16: 70-84.

3. Thomas P, Pang Y, Filardo EJ, Dong J. 2005. Identity of a membrane estrogen receptor coupled to a G-protein in human breast cancer cells. *Endocrinol.* 146:624-632.

4. Filardo EJ, Graeber CT, Quinn, JA, Resnick, MB, Giri D, DeLellis, RA, Steinhoff, MA, Sabo E. 2006. Distribution of GPR30, a seven-membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathological determinants of tumor progression. *Clin Cancer Res.* 12: 6359-66.

5. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science. 307:1625-1630.

6. Albanito L, Madeo A, Lappano R, Vivacqua A, Rago V, Carpino A, Oprea Tl, Prossnitz ER, Musti AM, Ando S, Maggliolini M. 2007. G coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17β-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. *Cancer Res*. 67: 1859-1866

7. Vivacqua A, Bonofiglio D, Recchia AG, Musti AM, Picard D, Ando S, Maggiolini M. 2006. The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17β-estradiol and hydroxytamoxifen in endometrial cancer cells. *Mol Endocrinol.* 20:631-646

8. Smith HO, Leslie KK, Singh M, Qualls CR, Revankar CM, Joste NE, Prossnitz ER. 2007. GPR30: a novel indicator of poor survival for endometrial carcinoma. *Am J Obstet Gynecol.* 196: 386 e1-9

9. Clarke R. 1996. Animal models of breast cancer: their diversity and role in biomedical research. *Br Ca Res Tr*. 39: 1-6.

10. Kordon EC. 2008. MMTV-induced pregnancy-dependent mammary tumors: early history and new perspectives. *J Mam Gl Biol Neopl.* 13: 289-297.

Appendices.

None.

Supporting data (figures 1-3).

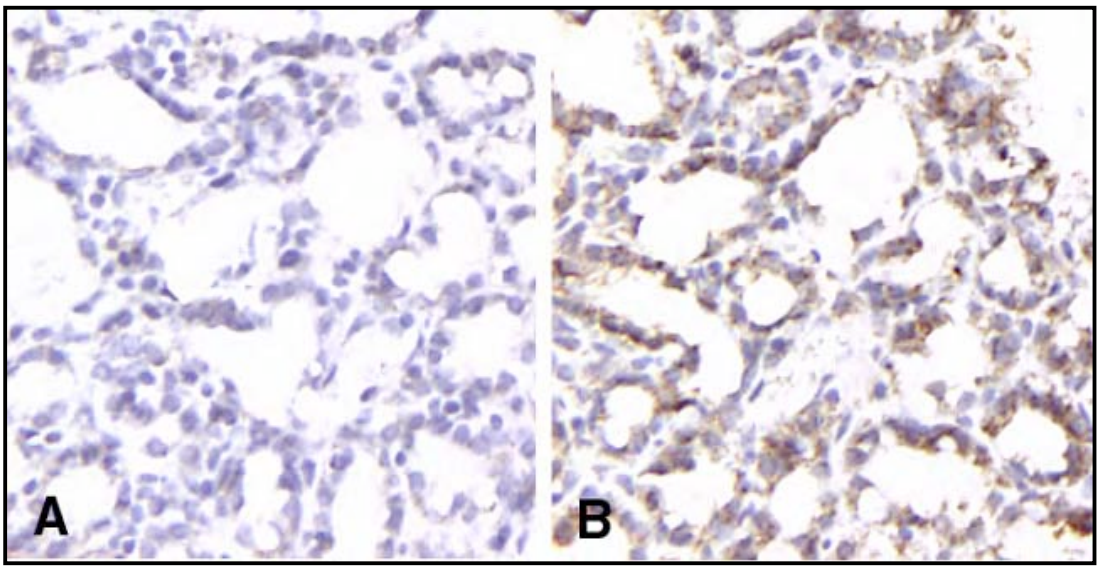

Figure 1. Failure to express HA-GPR30 protein in mammary glands from MMTV-HA-GPR30 mice. Mammary glands derived from HA-GPR30 transgene positive mouse, T12-1G, that was immunostained with (A) HA-antibodies or (B) C-ter GPR30 peptide antibodies. Images are shown at 100X.

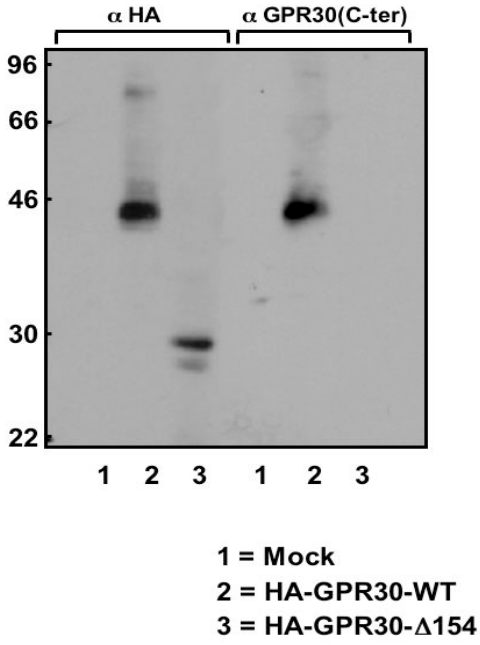

Figure 2. Expression of HA-GPR30 protein. Total protein (25 μg) from HEK-293 cells transfected with: vector, HA-GPR30, or C-terminally truncated HA-GPR30 immunoblotted with anti-hemagluttinin (HA) or GPR30 C-TER peptide antibodies. Molecular mass standards are indicated at left (kDa).

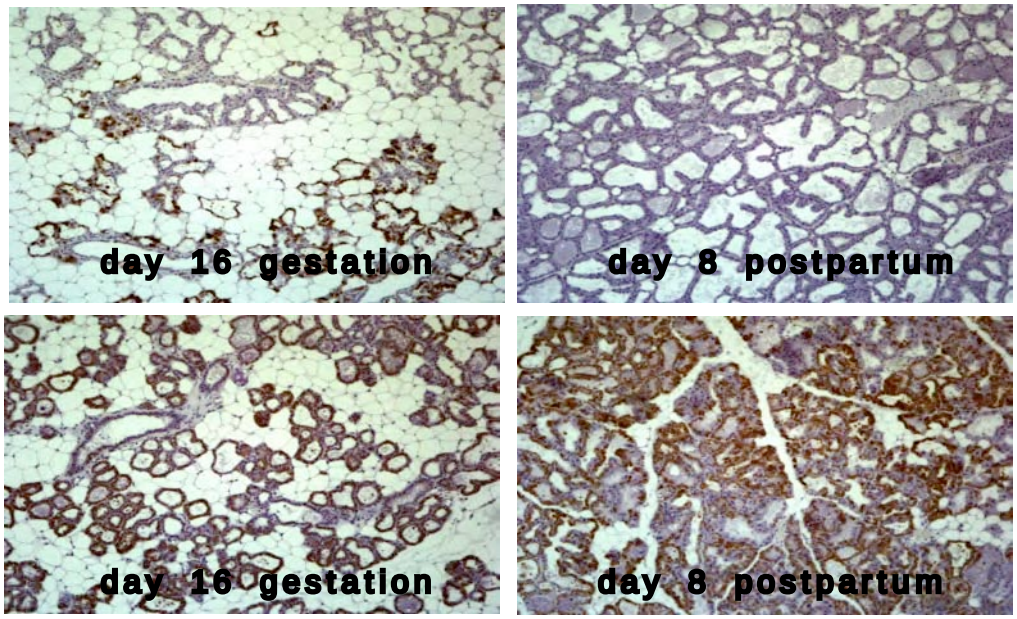

Figure 3. Expression of HA-GPR30Δ154 carboxyl-terminated protein in mammary glands from WAP-HA-GPR30Δ154 mice. Mammary glands from 4 individual transgene positive WAP-HA-GPR30Δ154 mice from the same founder on day 16 of gestation and from day 8 postpartum immunostained with HA antibodies. While transgene expression is strong, notice the variation in the percentage of mammary duct lobules that positively express the transgene product.