AD_____

Award Number: DAMD17-00-1-0311

TITLE: Functional Characterization of the Murine Homologue of PLAB, a Novel TGF-B Member, in Placental and Mammary Tissues

PRINCIPAL INVESTIGATOR: Guy S. Eakin

CONTRACTING ORGANIZATION: The University of Texas
M.D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: June 2001

TYPE OF REPORT: Annual Summary

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2001	Annual Summary (1 Jun 00 - 31 May 01)

4. TITLE AND SUBTITLE
Functional Characterization of the Murine Homologue of PLAB, a Novel TGF-B member, in Placental and Mammary Tissues

5. FUNDING NUMBERS
DAMD17-00-1-0311

6. AUTHOR(S)
Guy S. Eakin

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

E-Mail: ge691967@bcm.tmc.edu

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. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

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

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The goal of this proposal was to generate and analyze mice mutant for the *mPlab* gene. The gene was successfully cloned and sequenced. The targeting vector for production o: null embryonic stem cells was constructed. Embryonic Stem (ES) cells containing the conditionally null allele of *mPlab* were created. While testing these ES cells for correct homologous recombination events, we discovered that the *mPlab* gene had already been knocked out by another laboratory. Due to a lack of phenotype reported by the otl paper, we terminated our *mPlab* experiments and have begun designing a different set of specific aims. Here we detail the work generated under the *mPlab* studies, and present mini-proposal of our new research testing the mouse as a model for primate early development. If the reviewing board feels funding can be continued on the new researcl will submit a full proposal at its request.

14. SUBJECT TERMS
Mouse model system, ES cells, Placenta, Gene targeting, Embryonic Development, Transgenic mice, non-human primate embryology

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The relationship of extraembryonic membrane function to the progression of cancers, specifically cancers of the breast, is an understudied area of research. By understanding the factors that control the molecular architecture of the placenta and other fetal membranes, we hope to shed light on mechanisms by which placental function is established. The initial goal of this research had been to characterize the role of the gene *mPlab* in mice conditionally null for the gene. *mPlab* is a member of the TGF-β superfamily of transforming growth factors, and had been previously demonstrated to be expressed at highest levels in the developing placenta (1-5). Midway through our research, we discovered that another laboratory had published the phenotype of the *mPlab* deficient mice (6). In light of this, we were forced to abandon our research on *mPlab* and find other areas of fetal membrane research to explore. In this report, we detail the completed work on *mPlab*, and submit a mini-proposal of the new research to the reviewing boards under the understanding that, when and if necessary, a complete proposal of the new research will be submitted.

BODY.

The goal of our project was to characterize the loss of function of MPLAB in transgenic mice. In the May 2000 issue of Molecular and Cellular Biology, Se-Jin Lee's laboratory at Johns Hopkins University School of Medicine reported successful generation of a null allele of this gene and the resultant phenotype (6). Unfortunately, we did not see this report for several months because the gene had been published under a new name, *gdf-15*, and not *mPlab*, or any of several other names under which previous literature had been published. Though these names appeared in the body of the text, they did not appear in the abstract or keywords. Because of this, bi-weekly electronic database searching of recently published literature failed to return this reference. In late December, 2000, a discussion with a colleague who had knowledge of the published work led us to the finding that our research efforts had been previously published.

The findings of the Lee laboratory were that mice lacking GDF-15 had no apparent phenotype in any of the nearly 750 mutant animals analyzed. At this point, we had created our own null alleles of this gene, and had introduced them into embryonic stem cells. Because of the Lee lab's several month lead on our own research and difficulties inherent in tracking down subtle mutant phenotypes (7,8), we were forced to abandon this project. With regards to the Statement of Work supplied with our original DOD funding application, we have successfully completed approximately half of the work contracted with the Department of Defense. We have independently cloned and determined the sequence of *mPlab*(*gdf-15*), constructed a targeting vector to generate a conditionally null allele of the gene, and generated transgenic embryonic stem cells from that vector.

Our research over the intervening time has shifted to the development of an entirely new set of specific aims which seeks to test the mouse model as a legitimate model for primate embryology, especially with regard to the early establishment of the extra-embryonic membranes. Extraembryonic membrane biology was also the focus of the *mPlab* (*gdf-15*) study.

In speaking with Kathy Dunn at the DOD, we were led to believe that if we were to submit a description of our new research that the possibility existed that funding for DAMD17-1-0311 could continue for the new project. What follows is a summary of completed work on the original project and a mini-proposal justifying our new line of research. Should this be acceptable for further review, we will supply the necessary support documentation.

The research accomplishments associated with the original statement of work are as follows. Task 1 had been to determine the expression pattern of *mPlab* in wild-type mice. At this time we were concentrating fully on producing the targeting vector and generating targeted ES cells. As such, we elected to postpone the duties under task 1 until after ES cells had been successfully created and injected into mouse blastocysts. With respect to task 2, the cloning and determination of the genomic sequence of murine *plab*, we had successfully completed the cloning and had sequenced 15 kilobases surrounding the gene for use in future experiments. This information is unpublished. With regard to task three, the generation of the mutant mice, we had spent the first five months of the funding period creating the targeting vector detailed in the original statement of work and the genomic probes that would be necessary for genotyping the targeted ES cells and mutant mice. The creation of the targeting vector proved to be problematic due to an early error in determining the restriction map of the cloned gene. Once completed, however, the vector was completely sequenced and electroporated into embryonic stem cells. 400 AB1 ES cells were isolated and tested for correct targeting by Southern hybridization. Due to personal inexperience with the technique, the first two attempts were suggestive of a correct targeting event, but were inconclusive. Before we were able to retest the genotype we discovered the existence of the *gdf-15* knock-out paper (6). Shortly thereafter, we terminated the existing experiments, and began designing a new line of research.

What follows is a short description of our planned research involving non-human primate (baboon) embryos.

The basic premise of this research is that we, as a mouse research laboratory, use the mouse as a model to study early embryonic development for the ultimate purpose of understanding development in other mammalian species, specifically the human. The natural history of the mouse, as well as the availability of

multiple congenic lineages, have made it particularly attractive as a genetic tool for research in numerous biological phenomena. The underlying assumption is, however, that the mouse closely models the biology of other animal systems. The reality of the matter is that there are numerous documented cases where research in the mouse or human does not faithfully predict the biology of the reciprocal species (9).

This is probably most carefully studied in pharmacokinetic literature, but is increasingly evident in other fields. A review of recent literature reveals mouse-human differences in the biologies of menopause (10), breast cancer (11,12), ovarian function (13), and wound repair (14) among others (15-17).

In my specific interest of developmental biology, it is known for example that many of the functions carried out by human placental estrogens, are performed in mice by a wholly dissimilar class of molecules, the prolactins. The mouse placenta is, in fact, incapable of producing aromatase, an enzyme necessary for production of estrogens. Promoter studies of the human aromatase gene, however, have shown that elements of the human aromatase promoter can drive reporter gene expression in the murine placenta (18). This suggests that although the mature organ biology is quite different, the underlying molecular architecture of the two species may still be quite conserved. There exists, therefore, a class of placental research for which the tools available to murine biologists may provide significant advances in understanding of primate placental development and function. For these same reasons, however, it is necessary for mouse researchers to test the fidelity of their models in other species in order to assess where the limitations of the mouse may lie in any given field of research.

Here we present a project centered on the observation that primate and murine gastrulae display markedly disparate morphologies (Fig.1). This, of course, leads to the question of whether the differing morphologies are of similar molecular determination, or whether they are uniquely derived with respect to one another. The initial phase of the project is to define primate embryonic structures in molecular terms by examining expression patterns of murine molecular markers in primate gastrula-staged embryos.

Once molecularly similar tissues have been defined, we will begin looking at expressions of developmentally important molecules in order to screen for regulatory differences between murine and primate homologues.

In our first pass, we have chosen five gene products to examine based on their ability to robustly highlight features of each primary germ layer in the mouse embryo.

The *Oct-4* gene is a discrete marker of the epiblast, which begins expression in the preimplantation mouse embryo, and continues expression into the somite stages. *Oct-4* has been shown to be expressed in human preimplantation embryos

(19) , and might therefore be assumed as a reasonable positive control for testing our laboratory's ability to work with primate embryos. *Oct-4* is also expressed in the fetal and adult testis, which will allow us to test our antibody on adult tissues prior to using the antibody on embryonic tissue.

Amnionless is strongly expressed in the visceral endoderm of developing mice, and adult expression is detectable in mouse kidney (20).

Brachyury is a well known marker of the primitive streak mesoderm (reviewed extensively (21) and others. We are currently testing the possibility that *Brachyury* may be expressed in the adult murine brain.

Although we do not have antibodies for T-box proteins 4 or 5, they can be detected by *in situ* hybridization in the extraembryonic mesoderm of gastrulating mice (22). This expression begins during mesoderm induction and continues into the fetal period. Additionally, adult expression is detectable in the testes. Concurrently, we are establishing murine expression data for each of these markers. Although most have been previously published, the dynamics of their expression in the narrow window around gastrulation (23) are generally unpublished and must be demonstrated prior to use as tools for inter-species comparison. Pending the outcome of our "first pass" and the availability of embryonic tissue, other tissue markers will be included. These other markers include other endodermal markers such as *eem1* (24), or *dab2* (25) and other mesodermal markers such as *flt* and *flk* (26).

We are currently entering collaborations with researchers at the Kenyan Institute of Primate Research who will supply the embryos necessary for this research. Ideally, we would use minimal sample sizes of 3 embryos per marker per stage, and would determine any given expression pattern in serial sections through single embryos. In reality, it will be possible to reduce the number of embryos required by determining expression patterns of 2 markers per embryo by performing the analyses and controls in alternating sections.

With regards to staging, I have relied on two primary references, O'Rahilly and Müller, 1987 (27), which uses the Carnegie staging system, and Hendrickx 1971 (28), which uses the similar Streeter staging system.

If the literature behind the murine marker genes listed above is converted to Carnegie or Streeter staging, I would describe the expressions as follows. *Oct-4* is expressed from stages III through fetal stages. The *Amnionless* gene is expressed from stages IV-IX. *Brachyury*, *Tbx4* and *Tbx5* are expressed from stages VI through the fetal stages. Since mesoderm formation in the baboon begins prior to the appearance of the primitive streak, it is prudent to check for *Tbx4* and *5* expression in stages earlier than stage VII, the onset of *Tbx4* and *5* expression in the mouse.

For our purposes, we would like to examine expression patterns in stages V-VII. This equates to 3 stages for *Oct-4* and *Amn*, and 2 stages for *Brachyury*, *Tbx4* and *Tbx5*. At samples sizes of n=3, the total number of embryos needed is 3 stage V, 12 stage VI, and 12 stage VII, plus two extra embryos per stage to use in case of damage to the primary embryos. This is a total of 25 embryos.

This, of course is the ideal situation, we believe we can accomplish our goals with half the listed number of embryos per stage. This would bring totals to 2 stage V embryos, and 6 each of stages VI and VII, plus one embryo per stage to use in case of damaged embryos. This reduces the previous total to 17.

The purpose of this first pass is to test our ability to molecularly characterize the early baboon embryo. Molecular characterization will allow us to compare murine and baboon development where morphological similarities are not evident. This information, once supplemented by other markers and classical comparative anatomy studies will then be used as a reference to screen other developmentally important genes (Table 1) for spatial or temporal regulatory differences between the two species.

Any differences found would be classified as genes that could putatively bear responsibility for the morphological differences between primate and murine early development.

Ultimately, we would like to test these observed regulatory differences functionally in the mouse. In order to test this, the murine gene in question would be expressed in transgenic mice under the control of primate regulatory sequences. Our laboratory has significant experience in the production of such mice (please refer to the original "training environment" section of the DAMD 17-1-0311 funding proposal). Descendant embryos would then be scored for perturbed embryogenesis in the sense that we would be asking if these embryos displayed a morphologically more "primate-like" pattern of development. For instance, if a differentially regulated mesodermal gene were expressed in mice under baboon promoter sequences one possible outcome might be that extraembryonic mesoderm formation might begin earlier in the transgenic mice than in wild-type controls as defined by histology and expression of mesodermal markers.

There is precedent for these sorts of studies, Fougerousse et al. (29) have previously described examples of such mouse-human regulatory differences. In this paper they demonstrated differences in expressions of WNT7A, CAPN3, and LGMD2A in human embryos of Carnegie stages 12-21, but stopped short of functional tests due to technical limitations inherent to the candidate genes they chose.

In conclusion, what we outline here is an experiment designed to test the fidelity of the mouse as a model for primate early development by establishing a molecular

framework for comparing the early baboon embryo to similarly staged mouse embryos. Secondly, by screening primate homologues of developmentally important genes for regulatory differences between the two species we seek to identify genes that show the possibility of being involved in the establishment of the different morphologies between the gastrulae of the two species. Lastly, we will test possible involvements by observing the effects of murine genes under the control of baboon regulatory sequences in transgenic mice.

With respect to the numbers of embryos needed, we have taken a "best case scenario" approach and developed the proposal as if an inexhaustible source of primate tissue were available. We, of course, realize that this is not the reality of the situation, and are modifying the scope of the project accordingly in ongoing collaboration with the breeding colony

KEY RESEARCH ACCOMPLISHMENTS

* Cloning and sequencing of the *mPlab* gene
* Creation of a targeting vector for the production of a conditionally null allele of *mPlab*.
* Establishment of an ES cell lines containing the conditionally null allele of *mPlab* in a putatively heterozygous condition.

REPORTABLE OUTCOMES

There are currently no reportable outcomes for DAMD 17-1-0311, as defined by the examples in the "Research Technical Reporting Requirements"

CONCLUSIONS

An unfortunate consequence of the large scale and fast-pace of scientific research is that often multiple laboratories are working on closely related projects, and may be unaware of the other's efforts. This was the case with our research on the *mPlab* gene. In late December, 2000, we became aware that our effort to construct null alleles of the *mPlab* gene was a duplication of published work in another laboratory. It was apparent that continued research on *mPlab* would not be a productive endeavor for either the laboratory or the educational goals of the graduate student (G.E.) performing the research. As such we have designed a new project, submitted here in a mini-proposal, outlining a research program intended to test the limitations of the mouse system as a model for primate early development and disease. This research is a crucial step in the establishment of mouse models as numerous very basic differences between the biologies of mice and primates are accumulating in the literature. We have paid specific attention to the formation of the fetal membranes as an understudied organ with distinct morphological difference between primates and rodents. Nearly 30% of spontaneous abortion in humans and livestock is believed to be due to failures in the establishment of the embryonic membranes. In the case of reproductive assistance technologies, this statistic may double (30,31). In addition, the

relationship of the fetal membranes to progression of cancers is an understudied phenomenon. Cancer is diagnosed during 1 in 1000 pregnancies (32). As more and more women delay their first pregnancies into later years, the incidence of cancer diagnosis during pregnancy is expected to increase.

In addition to causing prolactin dependant density changes in the breast which affect the sensitivity of mammographies (32), the human placenta is a major source of steroid hormones, such as estrogen and progesterone, that affect the progression of many breast tumors. Mouse placentae, however do not produce estrogens. Our ability to bring the power of mouse research to bear against these questions rests, therefore, on our knowledge of the limitations to which the mouse can serve as a model for these diseases.

REFERENCES

1. W. D. Fairlie, et al. MIC-1 is a novel TGF-beta superfamily cytokine associated with macrophage activation. *J Leukoc Biol* **65**, 2-5 (1999).
2. M. Yokoyama-Kobayashi, M. Saeki, S. Sekine, S. Kato. Human cDNA encoding a novel TGF-beta superfamily protein highly expressed in placenta.*J Biochem (Tokyo)* **122**, 622-6 (1997).
3. V. M. Paralkar, et al. Cloning and characterization of a novel member of the transforming growth
factor-b/bone morphogenetic protein family.*J Biol Chem* **273**, 13760-7 (1998).
4. L. N. Lawton, et al. Identification of a novel member of the TGF-beta superfamily highly expressed in human placenta. *Gene* **203**, 17-26 (1997).
5. R. Hromas, et al.PLAB, a novel placental bone morphogenetic protein.*Biochim Biophys Acta* **1354**, 40-4 (1997).
6. E.C. Hsiao, et al. Characterization of growth-differentiation factor 15, a transforming growth factor beta superfamily member induced following liver injury. *Mol Cell Biol* **20** 3742-51 (2000).
7. C. Brayton, M. Justice, CA. Montgomery. Evaluating mutant mice:anatomic pathology. *Vet Pathol* **38**, 1-19 (2001).
8. P.A. Wood. Phenotype assessment: are you missing something? *Comp Med* **50**, 12-15 (2000).
9. A. Wynshaw-Boris. Model mice and human disease. *Nat Genet* **13**, 259-260 (1996).
10. F.L. Bellino. Nonprimate animal models of menopause: workshop report. *Menopause* **7**, 14-24 (2000).
11. A.D. Cardiff. Validity of mouse mammary tumour models for human breast cancer: comparative pathology. *Microsc Res Tech* **52**, 224-230 (2001).
12. S. Nandi. Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. *Proc Natl Acad Sci U S A.* **92**, 3650-3657 (1995).

13. B.S. Dunbar et al. The ovary as an immune target. *J Soc Gynecol Investig* 8 (1 Suppl Proceedings):S43-8 2001
14. A.B. Borisov. Regeneration of skeletal and cardiac muscle in mammals: do nonprimate models resemble human pathology? *Wound Repair Regen* **7**, 26-35. (1999).
15. C.M. Disteche. Escape from X inactivation in human and mouse. *Trends Genet* **11**, 17-22 (1995).
16. W.E. Wright and J.W. Shay. Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology. *Nat Med* **6**, 849-851 (2000).
17. S.V. Prasad et al. Structure and function of the proteins of the mammalian Zona pellucida. *Cells Tissues Organs* **166**, 148-164 (2000).
18. A. Kamat, K. H. Graves, M. E. Smith, J. A. Richardson, C. R. Mendelson. A 500-bp region, approximately 40 kb upstream of the human CYP19.*Proc Natl Acad Sci U S A* **96**, 4575-80 (1999).
19. C. Hansis, JA Grifo, LC Krey.Oct-4 expression in inner cell mass and trophectoderm of human blastocysts. *Mol Hum Reprod* **6,** 999-1004 (2000)
20. S. Kalantry.The amnionless gene, essential for mouse gastrulation, encodes a visceral-endoderm-specific protein with an extracellular cysteine-rich domain. *Nat Genet* **27**, 412-6 (2001).
21. V.E. Papaioannou, L.M. Silver. The T-Box gene family. *Bioessays* **20**, 9-19 (1998).
22. D.L. Chapan et al. Expression of the T-box family genes, Tbx1-Tbx5, during early mouse development. *Dev Dyn* **206**, 379-90 (1996).
23. K.M. Downs, T. Davies. Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* **118**, 1255-1266 (1993).
24.J.K. Divine. Expression of a novel antigen, EEM-1, in the mouse embryo and embryonic stem cell-derived embryoid bodies. *Dev Dyn* **218**, 207-11(2000).
25. E.E. Morrisey. The gene encoding the mitogen-responsive phosphoprotein Dab2 is differentially regulated by GATA-6 and GATA-4 in the visceral endoderm. *J Biol Chem* **275**, 19949-54 (2000).
26. B. Larrivee, A. Karsan. Signaling pathways induced by vascular endothelial growth factor (review). I*nt J Mol Med* **5**,447-56 (2000).
27. A.G. Hendrickx, *Embryology of the baboon* (University of Chicago Press, Chicago, 1971).
28. R. O'Rahilly and F. Müller, *Developmental Stages in Human Embryos*, (Carnegie Institution of Washington, 1987).

29. Fougerousse et al. Human-mouse differences in the embryonic expression patterns of developmental control and disease genes. *Human Molecular Genetics*, **9**, 165-173 (2000).
30. J.C. Cross, Z. Werb, S.J. Fisher. Implantaton and the placenta:key pieces of the developmental puzzle. *Science* **266**,1508-18 (1994).
31. A.J. Wilcox, et al. Incidence of early loss of pregnancy. *N Engl J Med* **319**, 189-95 (1988).
32. W.L. Donegan. Cancer and pregnancy. *CA Cancer J Clin* **33**, 194-214 (1983).
33. N.M. Antonelli et al. Cancer in pregnancy: a review of the literature: part I. *Obstet Gynecol Surv* **51**, 125-34 (1996).

APPENDICES

Fig. 1.

Fig. 1 Morphologies of Baboon (left) and Mouse Gastrula (right).
For certain structures such as the epiblast (blue) and visceral endoderm (dark green) comparisons may be made with reasonable certainty. Structures such as the extraembryonic mesoderm (pink), on the other hand, strain such simple morphological comparisons. While all murine mesoderm in produced by the ingression of epiblast tissues through the primitive streak, primate extraembryonic mesoderm is evident prior to the formation of the node or primitive streak. Other structures such as Reichert's membrane, depicted here as the outermost gray membrane of the mouse, have no morphological analog in the primate gastrula.

Table 1 – List of Candidate Genes for Screen for Potential Regulatory Differences

Extraembryonic Ectoderm	**Extraembryonic Mesoderm**
4311 Bmp4 Rex1 Mash2	Foxf1 Flt1 Tbx4 Tbx5
Primitive Streak	**Visceral and Definitive Endoderm**
Gsc Nodal Evx1 Brachyury	Tbx3 Dab2 Eem1 Mrg1 Hex Amnionless
Epiblast	
Wnt3 Oct4	

Budgetary issues.

The graduate student stipend of Guy Eakin, the Principal Investigator, has been the only money paid from this contract.