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AUTHORITY

USAMRMC, ltr, 1 Jun 2001.

AD_____

GRANT NUMBER DAMD17-94-J-4191

TITLE: Transgenic Rat Models for Breast Cancer Research

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REPORT DATE: October 1996

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1996	Annual (1 Oct 95 - 30 Sep 96)

4. TITLE AND SUBTITLE Transgenic Rat Models for Breast Cancer Research

5. FUNDING NUMBERS
DAMD17-94-J-4191

6. AUTHOR(S)
Anne E. Griep, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Wisconsin
Madison, Wisconsin 53706

8. PERFORMING ORGANIZATION REPORT NUMBER

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

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Oct 96). Other requests for this document shall be referred to Commander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, Frederick, MD 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The laboratory rat is an important model for studying breast cancer due to the many similarities in this disease between rats and humans. However, limited knowledge in manipulating the rat genome through transgenesis has prevented researchers from answering important questions in breast cancer research as well as in research on other human cancers. We proposed to carry out a series of detailed studies to optimize the many variables in transgenic manipulation, to extend transgenic rat technology to inbred rat strains, and to develop rat embryo cryopreservation. We have determined that removing glucose from the microinjection medium and adding cytochalasin for microinjection facilitate transgenic rat production. We have further developed a procedure for rapid and efficient cryopreservation of rat embryos that results in our ability to recover about half of frozen embryos as viable pups after transfer into pseudopregnant recipient rats. The particular DNAs we selected for our studies on optimizing transgenic rat production were ones that would likely generate new important rat models for breast cancer research. As a consequence of our many optimization studies, new transgenic rats models have been generated for addressing the roles of the *neu* and *ras* oncogenes and the growth factor TGFα in breast cancer.

14. SUBJECT TERMS Breast Cancer; Animal Models; Transgenic; Rats; Embryo Cryopreservation; neu Oncogene

15. NUMBER OF PAGES
22

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	Limited

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Ann D. Grieg 10/24/96

PI - Signature Date

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Please note: Page 2 (abstract), Pages 8-13 and 17-22 contain unpublished data and therefore should not be distributed.

5. Introduction

5a. General Background

Breast cancer is one of the leading cause of death among women, with one out of every nine women in the United States being predicted to develop this disease during her lifetime. As with all cancers, breast cancer is a disease in which numerous cellular and molecular genetic changes are thought to contribute to the multistaged progression of normal cells to a population of cells with unrestricted growth and metastatic potential. Over the last decade two classes of genes, cellular protooncogenes and tumor suppressor genes, have been identified as genes which play critical roles in regulating cell growth and differentiation. Deregulation of gene expression through chromosomal translocation or mutation in the regulatory elements of the gene, alterations in the activities of these gene products through mutation in the coding regions of the genes, or complete loss of these genes from the chromosome through mutation are considered to be mechanisms contributing to the failure of cells to maintain normal growth characteristics.

Both mice and rats have been extensively used as laboratory animal models in breast cancer research, as well as in cancer research in general. For several reasons, the rat is perhaps the more suitable of the two with respect to a model system for human breast cancer. Whereas a high percentage of breast cancer in the mouse is associated with the integration of the mouse mammary tumor virus (MMTV) into the *int*-1 locus with consequent deregulation of *int*-1 expression, there is no known viral etiology of breast cancer in rats, as in humans (1). Second, the progressive disease that leads to breast cancer in laboratory rats bears striking histological similarity to that seen in human breast cancer (2-4). Third, a high percentage of the resulting rat mammary cancers are hormonally responsiveness, closely mimicking that seen in human breast cancer. Finally, certain inbred strains of rats show susceptibility to breast cancer whereas others show resistance (5,6). Through genetic crosses between these strains, putative suppressors have been identified (7-9). This genetic susceptibility to breast cancer seen in the rat may bear similarity to the human disease where genetic predisposition is considered to be an important factor (10,11). In part for these reasons, the rat is accepted as the animal model of choice for screening chemopreventive drugs for human breast cancer therapy (2).

Transgenic mice have been widely used in breast cancer research. Mouse models have been developed in which the expression of deregulated *int*-1 (12), c-*myc* (13-16), activated H-*ras* (13, 17), activated c-*neu* (18-20), wild type c-*neu* (21), deregulated growth hormone (22), and deregulated transforming growth factor a (23-25) has occurred in mammary tissue. All cases lead to abnormalities in mammary epithelial cells ranging from epithelial cell hyperproliferation without tumor formation to tumor formation, apparently some being similar to ductal carcinoma in situ which is seen in human breast cancers. The most prevalent genetic alterations in human breast cancers appear to be amplification of the c-*neu* locus (26-28), found in approximately 20% of breast cancers, and mutations of p53 (10,11). Unfortunately, discrepancies between the phenotypes of the several activated-*neu* transgenic mouse models has resulted in the lack of a consensus as to the nature of the activities of the *neu* oncogene in mammary carcinoma in these models. A more promising result was obtained from investigators who analyzed transgenic mice with deregulated expression of the wild-type *neu* proto-oncogene in mammary tissue. These mice developed focal mammary carcinomas, but only after long latency. The loss of p53 function through gene knockout led to only a very low percentage of animals with mammary adenocarcinoma (1 out of 26 p53 null mice) whereas there was a high incidence of malignant lymphomas (20 out of 26). These studies provide the best animal models to date for studying the correlation between disruptions in expression or activities of these cellular

genes and the incidence of mammary carcinoma. However, they may not be truly reflective of the genetics, histopathology, or the progressive nature of human breast cancer.

Considering the depth of knowledge generated by previous studies of breast cancer in the rat and the striking parallels between the rat and human disease, the availability of transgenic rat technology would greatly enhance breast cancer research. Transgenic rats would provide an alternative, and perhaps more suitable, animal model for dissecting the molecular mechanisms of mammary carcinogenesis and testing putative therapeutic agents. In addition to providing good models for breast cancer, the rat has been widely used for biochemical and metabolic studies, owing to its larger size. A large portion of research in neuroanatomy and neurophysiology is based upon the rat. The rat is the animal in which the multistage nature of hepatocarcinogenesis has been established and studied (29). All these areas of research would profit immensely from the availability of transgenic rats.

Recently, the Transgenic Animal Facility at the University of Wisconsin Biotechnology Center developed the capacity to generate transgenic rats, primarily with the encouragement of two university colleagues, Dr. Henry Pitot, an expert in hepatocarcinogenesis, and Dr. Michael Gould, an expert in breast cancer. Through our initial attempts at transgenic rat production, we have successfully generated transgenic rats for each of these cancer researchers. However, the state of transgenic rat technology is rudimentary compared to that for transgenic mice and as such has received only limited use to date. Despite our initial successes, the production of transgenic rats is at present an extremely laborious task. As a consequence of the technical impediments we now encounter, the time and cost for generating transgenic rats is many fold higher than that for the generation of transgenic mice. For many investigators, this high cost is prohibitive. Thus, only with further improvements will this technology be as accessible for the generation of transgenic rats as it has been for the generation of transgenic mice.

Because we foresee a long term and expanding demand for transgenic rats, especially in the breast cancer research field, we propose an investigation designed to optimize transgenic rat production. This proposal to optimize transgenic rat technology was initiated because we believe that significant improvements can be made in both microinjection and embryo transfer techniques which would greatly facilitate transgenic rat technology. These advances should lead to the reduced cost in the production of transgenic rats, and to the capacity to generate transgenic rats in inbred backgrounds. Importantly, during the course of our optimization studies, a series of transgenic rat models for breast cancer research will be generated.

5b. Specific Aims and Statement of Work

Therefore, we proposed this infrastructure enhancement grant to provide a resource to the breast cancer research community for the generation of novel transgenic rat models for breast cancer research. The specific aims we proposed are:

(1) To generate transgenic rat lineages specifically for breast cancer research and to make these transgenic rats readily available to the breast cancer research community at a reasonable cost.

(2) To determine the most efficient technical procedures for the rapid generation of transgenic rat lineages on an outbred genetic background and on inbred genetic backgrounds appropriate for breast cancer research.

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(3) To develop efficient procedures for rat embryo cryopreservation.

(4) To develop and maintain the necessary resources and establish procedures for ongoing data sharing and communication amongst transgenic rat laboratories and with breast cancer researchers.

To accomplish these specific aims, we developed a Statement of Work that incorporated aspects of all four specific aims into each of two chronological stages. Stage One dealt with the optimization of technologies for transgenic rat production and cryopreservation using outbred rat strains and Stage Two with optimization for transgenic rat production and embryo cryopreservation using inbred rat strains. The first stage of the Statement of Work, designed to cover years 1 and 2 of the grant period, included the following points:

(a) Using MMTV-*neu*^{WT} and MMTV-*neu*^{mut} as test DNAs, optimize variables in microinjection and embryo transfer in the outbred Sprague-Dawley background.

(b) Maintain a small breeding colony of the *neu* transgenic rats (6 lineages) for dissemination to other breast cancer researchers.

(c) Develop embryo cryopreservation for Sprague-Dawley rat embryos. Cryopreserve *neu* transgenic rat lineages.

(d) Solicit requests for DNAs from the breast cancer research community. Have advisory board choose DNAs, judged to be of the greatest potential value to breast cancer research, for microinjection during years 3 and 4.

(e) Develop and make available to transgenic rat and breast cancer research communities specialized information databases.

In this second year of the grant award, we addressed each of the Specific Aims listed above in the context of performing Points (a), (c), and (e) itemized above in the Statement of Work, Stage 1. However, our overall progress during the second year was significantly hampered by three major problems. First, Dr. Jan Lohse, who had been performing all of the rat embryo microinjections, terminated employment in July of 1995. Thus, efforts to fine tune microinjection techniques and to generate new transgenic rat lineages for breast cancer research were put on hold until a suitable replacement for Dr. Lohse could be found. Second, the Transgenic Animal Facility moved its laboratory, microinjection facility and animal facility into the new Biotechnology Center/Genetics building in August of 1996. During this move, microinjections were temporarily suspended as time was required to reestablish the facility. Not unexpectedly, problems with the functioning of the animal facility occurred which required time to solve. Third, Kathy Helmuth, who is responsible for establishing the procedure for rat embryo cryopreservation was on maternity leave for two full months and then worked at 60% time for an additional two months. During this period, work on rat embryo preservation was temporarily suspended.

We have successfully overcome each of these hurdles. First, after an extensive search and waiting period, we were able to replace Dr. Lohse with Dr. Joe Warren. Dr. Warren arrived May 1, 1996. Dr. Warren has had extensive experience with production of transgenic rats in his former place of employment, VPI Research in Cambridge, UK and with production of chimeric mice for gene targeting experiments. Dr. Warren has successfully reestablished the transgenic rat program in our facility and is progressing towards the generation of transgenic rats with a new DNA of potential value to breast

cancer researchers. Second, we are now comfortably settled in our new space. All problems with the physical settings have been solved. Thirdly, Ms Helmuth has returned to full time work and has recently made significant progress in finalizing the procedure for rat embryo cryopreservation. Thus, progress towards achieving the goals of this grant are now back on track. The ensuing body of this progress report summarizes our work on the individual aims of this grant over this past year.

6. Body

6a. Statement of Work Point A (Specific Aim 1): Using MMTV-*neu*^{wt} and MMTV*neu*^{mut} as test DNAs optimize variables in microinjection and embryo transfer in outbred Sprague-Dawley background: Generation of transgenic rats carrying MMTV-*neu*^{wt} and MMTV-*neu*^{mut} DNAs.

An essential aim of this grant is to generate new valuable transgenic rat strains for breast cancer research. During the first year of this grant, we generated numerous transgenic rat lineages with several DNAs of interest to breast cancer research. These DNAs were as follows: First, MMTV-*neu*^{wt}, which consists of a mouse mammary tumor virus long terminal repeat driving expression of the wild type neu protooncogene. The MMTV promoter sequences have been demonstrated to drive expression of linked genes to the mammary epithelium of transgenic mice (19-21) and, hence, would be expected to do so in the rat as well. The neu oncogene has been shown to be a frequently mutated gene in human breast cancers (26-28). Thus, a rat model where high levels of wild type neu would be expressed should be of value in evaluating the role of this protooncogene in breast cancer. Furthermore, such a rat model could be used in studies to evaluate the role of carcinogenic agents as cofactors in neu-associated breast cancers. The second DNA, called MT-*neu*^{mut} we chose to use is one where a mutated neu oncogene is fused to the mouse metallothionein promoter which is inducible by heavy metals such as zinc (22,23,25). The inducible approach was chosen to express the activated oncogene because of the worry that if expression of a mutated oncogene occurred too early in the life of the rat, stable rat lines would never be derived. The final DNA, called H*ras*-K*ras* consists of the transcriptional control regions of the H-*ras* gene fused to the coding sequences of the K-r*as* gene. Activated H-r*as*, but not K-*ras*, is frequently found in rat mammary carcinomas arising as a consequence of treatment with carcinogens (13,17). This transgene DNA is one of a series of transgenes designed to study the mechanisms whereby this differential activation occurs following carcinogen treatment. Over the course or year two of this grant, these transgenic rat lineages have been under study in the laboratory of Michael Gould in the Human Oncology department at University of Wisconsin Medical School. Although we had intended to maintain a small breeding colony of these rats for other investigators in the breast cancer research community to use, we delayed in these efforts until Dr. Gould's lab has completed the initial characterization of these transgenic rats. After Dr. Gould's laboratory has finished their assessment of the effects of deregulated neu expression on mammary carcinoma, we will reestablish a small breeding colony for the purpose of disseminating these animals to other interested investigators in conjunction with cryopreserving these lineages (see Section 6c).

During the second year of this grant, we also began microinjection experiments to produce transgenic rats expressing high levels of transforming growth factor alpha (TGFα) from the MT promoter. As noted in the general background section, deregulated expression of TGFα in transgenic mice is associated with epithelial hyperplasia and subsequent carcinoma development (23-25). Given the close comparison of breast cancer

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in rats and humans, it should be of value to determine the potential effects of high TGFα levels on carcinoma development in the rat. Furthermore, with the use of an inducible promoter, these transgenic rats can be used to examine the effects of low-level deregulated expression as well as high level deregulated expression on the mammary gland. The generation of these MT-TGFα animals is in progress at the present time.

A goal listed for the second year of this grant was to solicit requests from the breast cancer research community at large for additional DNAs to use in the generation of transgenic rat models for breast cancer research (statement of work point d). We have delayed soliciting requests because our progress on the technical aspects of our work was impeded by the long time period of down time between the time Dr. Lohse left and Dr. Warren arrived. before the arrival of Dr. Warren, we were not at the appropriate point in our preliminary studies. Having now successfully reestablished our transgenic rat program, we anticipate that we will put forth the announcement for solicitation of proposals by the end of December of this year or beginning of January 1997.

6b. Statement of Work Point A (Specific Aim 2): Using MMTV-*neu*^{wt} and MMTV*neu*^{mut} as test DNAs optimize variables in microinjection and embryo transfer in outbred Sprague-Dawley background: Optimize variables in microinjection and embryo transfer technique.

The second aim of our studies is to investigate ways to increase the efficiency and ease of transgenic rat production. During the first year of this grant, we made significant progress towards evaluating the technical factors that influence the ultimate success in producing transgenic rats (refer to the progress report from year 1 for details on these experimental manipulations). In all cases, our goal was to determine how to get the highest percentage of transgenic rats with the lowest number of added manipulations to which the embryos were subjected during the experimental process. Through these initial studies, we established that a small modification in the culture medium composition, the omission of glucose from the conventional mouse M2 medium, resulted in somewhat higher embryo viability and significant increases in the numbers of overall transgenic rat production. Additionally, the use of cytochalasin B was observed to stiffen the embryonic membranes, making it easier to microinject. With respect to modifications to the embryo transfer aspect of the procedure, we determined that one gets optimal recovery of transferred injected embryos if between 40-50 embryos are transferred into each recipient Sprague-Dawley rat. We also determined that the use of epinephrine is beneficial during the transfer procedure to reduce bleeding from the numerous blood vessels that ramify through the ovarian bursa.

During the second year of this grant, with the arrival of Dr. Warren, we have adopted as routine the use of M2 medium lacking glucose, transfer of approximately 45-50 embryos per recipient including unmanipulated carrier embryos and the use of epinephrine. Data from Dr. Warren's recent experiments is presented in Table 1. In Dr. Warren's hands the use of these modifications has resulted in 9.4% of transferred embryos surviving to birth. This rate of embryo survival now approximates the typical survival rate in mouse microinjection experiments. Dr. Warren is proceeding to reevaluate the use of cytochalasin, to establish if, in his hands, this reagent enhances the percentage of embryos surviving microinjection.

6c. Statement of Work Point C (Specific Aim 3): Develop efficient methods for cryopreservation of Sprague-Dawley rat embryos.

As mentioned in background, we have established previously efficient methods for the cryopreservation of transgenic mouse embryos. It is clear that adaptation of this

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technology to the rat will be critical for the long term success of transgenic rat programs in our facility as well as world-wide. From our experience with cryopreserving hamster embryos performed in collaboration with the laboratory of Dr. Barry Bavister at the University of Wisconsin, we could not assume that procedures for efficiently cryopreserving rat embryos would be identical to those used for mouse embryos. When we began our work to develop techniques for efficient cryopreservation of rat embryos, very little data were available in the literature; the only literature sources coming from Japanese groups who performed there studies exclusively with the Wistar strain of rats. (30, 32).

The freezing and thawing process requires that one be able to dehydrate the embryo with a cryoprotectant before freezing and then rehydrate the embryo after freezing without losing viability of the embryo. There are multiple variables that could affect embryo viability. During the first year of the grant, we began a systematic evaluation of all the steps in the cryopreservation process using the outbred Sprague-Dawley rat as the strain of choice. During this past year, we have completed our studies to evaluate the requirements for retaining high viability of rat morulae during the cryopreservation process. To efficiently assess the suitability of each of multiple variables in the cryopreservation protocol, we used the ability of morulae to develop to blastocysts in culture as the criteria for determining if a given agent or manipulation used in the cryopreservation procedure would support embryo viability. We demonstrated that HECM-1 containing 7.5 mM glucose was the optimal medium for preserving viability of rat morulae (Table 2) and that the inclusion of amino acids in the culture medium does not significantly enhance embryo viability (Table 3). These results confirmed the observations of Miyoshi et al. (30) and were consistent with the knowledge gleaned from work on hamster that glucose is necessary for development from 8 cell to blastocyst stage. In agreement with Miyoshi, et al. (30), we determined that an osmolarity between 244 and 264 mosmoles was ideal (equivalent to 80 to 88 mM NaCl) for supporting embryo viability. We determined that glycerol is a suitable cryoprotectant for use with rat embryos, as had been demonstrated by Kasai (32) and that an appropriate regimen for dehydrating and rehydrating rat morulae was exposure to 1.5 M glycerol for 15 or 20 minutes followed by rehydration directly in 0.3M sucrose for 10 minutes or rehydration in 1.5M glycerol/0.3M sucrose for five minutes followed by 0.3 M sucrose for five minutes (See Fig. 1). Our data indicate no statistically significant difference between these different conditions if a minimum number of embryos per 100 µl culture drop of 13-18 is maintained. If the numbers of embryos per 100 µl culture drop is as low as 10-12, then the more rapid rehydration procedure is statistically more detrimental to embryo survival than the slower rehydration process. Therefore, a minimum embryo density appears to be important for maximal survival. Because we performed these manipulations initially at room temperature and ultimately the freezing procedures require that embryos be exposed to the dehydration solution at 4^0C prior to the actual freezing, we determined if this lower temperature had any negative impact on embryo viability in the dehydration or rehydration medium (See Fig. 1). Exposure to cryoprotectant at 4^0C did not reduce embryo viability. We tested various starting points for cooling, including moving embryos from room temperature to -4°C directly, moving embryos onto ice prior to immersing in -4°C methanol bath and placing embryos on ice then slow cooling starting at +4°C rather than at -4°C. Since our controlled-rate freezing unit has the capacity to cool only to -50°C rather than -75°C, initially we chose -50°C as our end point for slow cooling and placed frozen embryos into liquid nitrogen at that point. Subsequently, we determined that cooling to -35^0C, as is routine for mouse embryo cryopreservation, is adequate to support embryo viability. Using -35^0C as the end cooling temperature rather than -50^0C (or -75^0C) saves substantial time. However, when transferring straws from the methanol bath to storage in liquid nitrogen containers, we found it necessary to submerge the straws in a dewar of liquid nitrogen before transferring into the canisters in the liquid nitrogen bath. This step may help to assure that embryos are

sufficiently cold when that the transfer to the canisters from the liquid nitrogen storage tank occurs so that the embryos do not warm up to temperature that is detrimental for embryo survival while being moved into position in the liquid nitrogen tank. It is possible that were cooling to be continued down to -50^{0}C or -75^{0}C, this step may not be as crucial as we have found it to be.

At the time of last years report, we had determined that we could revive live embryos after subjecting them to our freezing and thawing regimen. Over the past year we have frozen and thawed hundreds of rat morulae. Unfortunately, our early data indicated that the viability upon thawing was highly unpredictable. While in about 2/3 of cases, we would find a high rate of survival after thawing (up to greater than 90% of embryos in a straw), about 1/3 of the time, virtually all embryos in a given straw would be nonviable. Within the last few months, we have identified the reason for these inconsistent results. We've determined that the embryos must be completely submerged in the methanol bath to retain viability. In typical use of the BioCool freezer, straws containing embryos are positioned in a rack that suspends them in the methanol bath. Unfortunately, the design of this holder is such that it is not possible to know if a straw is perfectly positioned so that the embryos are completely submerged. Simply submerging the straws directly into the methanol bath without the use of the holder avoids this problem. With this modification, we consistently obtain greater than 90% viability of embryos in each and every straw.

Thus, one of our major accomplishment over the past year has been that we've demonstrated that we can freeze and thaw rat morulae with greater than 90% viability. All of the above mentioned studies were performed in vitro and our end point was invariably viability upon thawing or the ability to develop from morulae to blastocyst in culture. Ultimately, however, the goal of any cryopreservation protocol is to have the ability to regenerate live animals from frozen embryos. Our final goal over the last year has been to demonstrate that we could successfully revive live rats from frozen embryos. To that end, we first determined our baseline rate of live births from unmanipulated rat morulae transferred into the uterine horns of pseudopregnant recipients. In these experiments, rat morulae were isolated from day 4 p.c. Sprague-Dawley donors and transferred into either day 3 or day 4 recipients. Table 4 shows that 77.8% of unmanipulated embryos transferred into day 3 recipients were recovered as live pups whereas only 39% of unmanipulated morulae transferred into day 4 recipients were recovered. Thus, we chose to use delayed recipients as hosts for frozen/thawed embryos. We transferred over 200 frozen/thawed morulae into delayed recipients and recovered 84, or 46.2%. Rate of pregnancy was high (86.7%). Finally, we determined if embryos derived superovulated donors can be cryopreserved with the same efficiency as those generated by natural matings. Our data, shown in table 4, indicates that embryos from superovulated females behave similarly to those iasolated from naturally mated females. Therefore, our cryopreservation protocol allows us to recover 60% of the expected numbers of embryos as live pups.

Thus, over the past year, we have fine tuned our cryopreservation protocol so that approximately 90% of embryos appear viable upon thawing and half of those can be recovered as live pups. Presently, a manuscript is being prepared describing our procedure. In the Statement of Work, we indicated that we would begin cryopreserving the MMTV-*neu* and MT-*neu* transgenic rats during the second year. At present time, we have arranged with Dr. Gould to begin this process as soon as Dr. Gould's laboratory has a suitable cohort of proven transgenic males for us to use. We anticipate that within the next 4 weeks, we will have begun cryopreserving these valuable transgenic rat lineages.

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6d. Statement of Work Point C (Specific Aim 4): Develop and make available to transgenic rat and breast cancer research communities specialized information databases.

The ability to communicate easily, effectively and efficiently with others in transgenic research and breast cancer research is essential in today's rapidly moving scientific world. To this end, many find it useful to communicate using the Internet where bulletin boards are reaching their highest popularity. In the first year of this grant, we joined two "clubs" on the Internet: Rodent Research and Embryo Mail. Over the second year, we have continued to be involved in dialogue through these electronic means and continue to find these services effective vehicles for rapid and informal discussion with our colleagues.

An extensive, up to date, easy to use directory of transgenic animal researchers and breast cancer researchers who use animal models as their primary model system is necessary to facilitate communication between these large groups of investigators. A member of the University of Wisconsin Biotechnology Center's outreach team will be assisting us with this effort. This directory is being designed so that updates (additions, deletions) can be made easily. Our outreach member is assisting us is to generate a broad-based literature search strategy which can be used at a monthly interval to retrieve relevant citations from the vast amount of current primary literature. On a less frequent basis, this strategy will enable us to search different and more costly sources of information such as those pertaining to patent-related materials.

Within the transgenic animal research community, there are several databases, such as the TBASE database operated by the Johns Hopkins University, which aim to list most knockout mice and transgenic animals in existence. These groups have elected to make as their first priority accumulating all of the information on the knockout mice. Thus, the effort to accumulate transgenic mouse and especially transgenic rat data is secondary. Accomplishing our task will then enable us to devise a current listing of all transgenic rodent models for breast cancer research as well as all transgenic rat models. This database will be maintained and updated periodically so that at the end of the grant period, the current information can be transferred to larger transgenic database units in this country such as TBASE or similar databases.

7. Conclusions

During the past year, we have made excellent progress in achieving our goals which are to generate transgenic rat models for breast cancer research, improve the efficiency and ease with which transgenic rats are produced, to develop effective methods for cryopreservation of rat embryos and to develop the capacity for state of the art communication between the University of Wisconsin Transgenic Animal Facility and the transgenic rat and breast cancer research communities. After losing the individual who had been responsible for transgenic rat microinjections, we successfully recruited a talented individual, Dr. Joe Warren, to take over this work. Dr. Warren has reestablished our transgenic rat program and is currently generating a new series of transgenic rats with potential value to breast cancer researchers, the MT-TGFα rats. All indications are that Dr. Warren, using the information gleaned from our previous year's careful evaluation of the procedure, is poised to have increased substantially the ease with which transgenic rats can be generated, at least on the outbred Sprague-Dawley background. Secondly, we have succeeded in developing an efficient straight forward rat embryo cryopreservation protocol that results in the recovery of a high percentage of cryopreserved embryos as live pups. We

are preparing a manuscript on this procedure. We are in the process of cryopreserving two different strains of transgenic rats of value to the breast cancer research community. Lastly, we have begun to generate a current directory of transgenic rat researchers and breast cancer researchers using animal models in their work. This should help increase everyone's awareness of the state of the fields and facilitate communication between workers with overlapping research goals.

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9. Appendix

Table 1: "Survival of Microinjected Rat Embryos"

Table 2: "In Vitro Development of Rat Morulae Using Different Culture Media"

Table 3: "Effects of Amino Acids on Embryo Viability"

Figure 1: "Effects of Glycerol and Sucrose Exposure on Blastocyst Development"

Table 4: "Viability of Frozen/Thawed Morulae After Transfer into Pseudopregnant Females"

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Table 1. Survival of Microinjected Rat Embryos

Medium	Number embryos surviving injection	Fraction (%) recipients pregnant	Fraction (%) transferred embryos born#
NG	1419	29/45 (64)	87/926 (9.4)

NG, mouse M2 medium lacking glucose

#, only embryos transferred to pregnant recipients are included in this calculation

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Table 2. In Vitro Development of Rat Morulae Using Different Culture Media

Culture Medium	No. Embryos Cultured	% Blastocyst at 42 hours
M16 + BSA	49	71.4
M16 +PVA	49	71.4
HECM-1	17	0
HECM-1 + 7.5mM glucose	49	79.6

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Table 3. In vitro development of frozen/thawed morulae

Culture Medium	No. Viable Morulae Recovered after Thawing/No. Frozen (%)	24 hours after thawing		42 hours after thawing
		No. Blastocyst	No. Compact Morula	No. Blastocyst
HECM-1 + glucose	31/34 (91%)	11 (35%)	15 (48%)	21 (68%)
HECM-1 + glucose + amino acids	30/34 (88%)	8 (27%)	13 (43%)	22 (73%)

Figure 1. Effects of Glycerol and Sucrose Exposure on Blastocyst Development

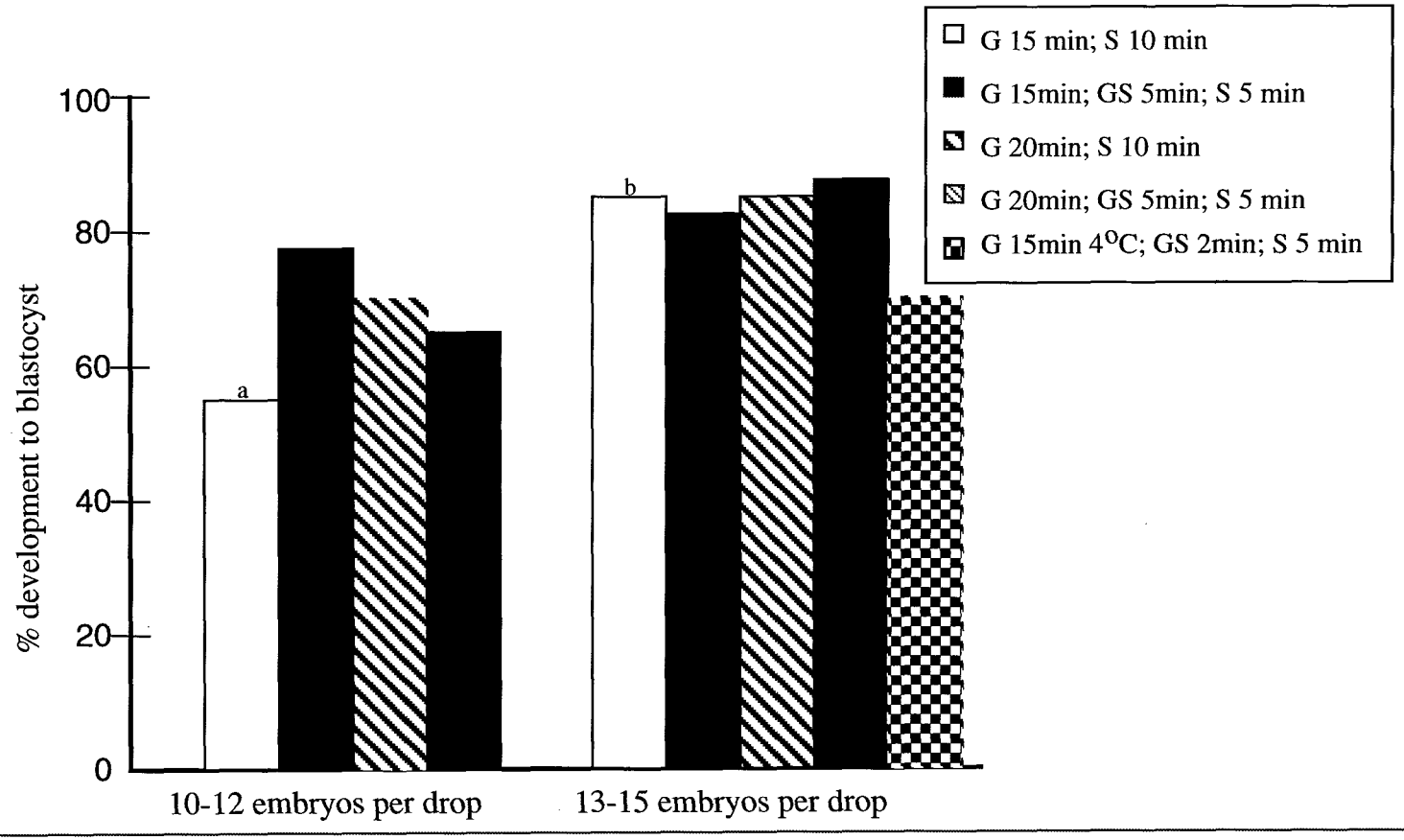

Fig. 1. Morulae were dehydrated in glycerol for either 15 min at room temperature (G 15min), 15min at 4°C (G 15min 4°C) or 20 min at room temperature (G 20min). Embryos were then rehydrated in either a two-step process:glycerol and sucrose for 5min then sucrose for 5min(GS 5 min, S 5min); or in a one-step process: sucrose 10min (S 10min). Morulae were cultured in HECM-1 (80mM NaCl) + glucose at 37°C in 5%CO_2 and devleopment to blastocyst recorded after 42 h. ^{ab}Different superscripts indicate significant differences ($p<0.05$.)

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Table 4. Viability of Frozen/Thawed Morulae after Transfer into Pseudopregnant Females

	No. Morulae Recovered After Thawing/No. Frozen (%)	No. Morulae Transferred	Fraction of Recipients Pregnant (%)	No. (%) Offspring Born *
Transfer of unmanipulated morulae into day 4 recipients	NA	43	2/3 (66.7%)	11 (39.3%)
Transfer of unmanipulated morulae into day 3 recipients	NA	54	4/4 (100%)	42 (77.8%)
Transfer of frozen/thawed naturally ovulated morulae into day 3 recipients	244/254 (96.1%)	218	13/15 (86.7%)	84 (46.2%)
Transfer of frozen/thawed superovulated morulae into day 3 recipients	27/28 (96.4%)	27	ND	ND

*Data for pregnant recipients only

ND, not determined

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Reports to be changed to "Approved for public release; distribution unlimited"

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