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MALIGNANT TRANSFORMATION OF DIPLOID HUMAN FIBROBLASTS BY TRANSFECTION OF ONCOGENES

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PART ONE -- PROPOSED STUDIES, 1990-1992
PART TWO -- PROGRESS REPORT, July 1986 - June 1989

ABSTRACT

Although there is good evidence that carcinogen exposure is a major cause of human cancer, it has proven impossible to transform normal human fibroblasts or epithelial cells in culture into malignant cells by treating them with carcinogens. This failure may reflect an inability to identify and isolate cells containing one or more premalignant changes so that these can be expanded and exposed to carcinogens a second time to induce additional required changes. A second serious roadblock to the sequential introduction of changes and expansion of clonally-derived cells containing such premalignant changes is the finite life span of human cells in culture. Using transfection of specific human oncogenes in a series of specially-selected vectors, we have overcome these obstacles and have recently succeeded in generating an infinite life span diploid human cell strain MSU-1.0, which appears to be normal in all other characteristics. From that cell a second cell strain, MSU-1.1, was generated which we have been able to transform into a malignant state not only by transfecting the cells with oncogenes but also by treating them with chemical carcinogens. We now have evidence that there is not just a single linear process which results in malignant transformation. Rather, cells appear to progress to malignancy on a series of parallel, sometimes overlapping tracks. We now propose to carry out detailed studies of the specific mechanisms of malignant cell transformation using the cell strains available in this laboratory to achieve the goal of building relevant quantitative models of carcinogenesis. We will conduct theoretically-based studies to obtain information as to just how various genotypic changes correlate with the various stages of transformation, and practical studies that take cells known to be one or at most three steps removed from being malignant transformation, and by using ionizing radiation treatment and appropriate selection techniques, isolating malignantly transformed cells.

FOREWORD

This document contains two parts. PART ONE outlines the new work to be pursued during the next three year period and gives the Budget, on pp. 40 - 42. PART TWO summarizes the reaserch progress made during the past three years, 1986-1989. The answers to the specific six items of information requested by the DOE for the reviewers are located as follows:

1) Main accomplishments: Part Two, Section IV, pp. 55-85.

2) New and continued objectives: Part Two, Section VI, p. 89; and Part One, Sections II and III, pp. 9-30.

3) Personnel trained: Part Two, Section VII, pp. 90-91.

4) Bibliography--associated with Grant: Part Two, Section II, pp. 42-50.

5) Significance: PartOne, Section I, pp. 6-8b.

6) Division of Federal support: Part Two, Section VIII, pp. 92-98.

PART ONE, pages 5 - 42, is intentionally omitted from this report.

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PART TWO

COMPREHENSIVE PROGRESS REPORT

DE-FG02-87-ER60524-01-03

I. PERIOD COVERED: JULY 1, 1986 - JUNE 30, 1989.

II. PUBLICATIONS RESULTING FROM RESEARCH SUPPORTED BY THIS GRANT.

A. Papers in refereed journals.

1. Y. Wang, S. Kateley-Kohler, V. M. Maher, and J. J. McCormick. ^{60}Co radiation-induced transformation to anchorage independence of fibroblasts from normal persons and patients with inherited predisposition to retinoblastoma. Carcinogenesis, 7:1917-1929, 1986. (Published in Nov. 1986 after the preceding Progress Report on the Grant Application had been submitted.)

2. J. J. McCormick, S. Kateley-Kohler, M. Watanabe, and V. M. Maher. Abnormal sensitivity of fibroblasts from xeroderma pigmentosum variants to transformation to anchorage independence by ultraviolet irradiation. Cancer Res. 7, 489-492 (1986).

3. P. A. Ryan, J. J. McCormick, and V. M. Maher. Modification of MCDB-110 medium to support prolonged growth and consistent high cloning efficiency of diploid human fibroblasts. Exp. Cell Res., 172:313-328, 1987.

4. P. J. Hurlin, D. G. Fry, V. M. Maher, and J. J. McCormick. Transformation of diploid human fibroblasts by the T24 oncogene. Cancer Res. 47:5252-5257, 1987.

5. J. J. McCormick and V. M. Maher. Towards an understanding of the malignant transformation of diploid human fibroblasts. Mutat. Res., 199:273-291, 1988.

6. D. G. Fry, P. J. Hurlin, V. M.Maher, and J. J. McCormick. Transformation of diploid human fibroblasts with the v-sis, PDGF2/c-cis or T24 H-ras genes. Mutat. Res. 199, 341-351 (1988).

7. J. J. McCormick, D. Yang, V. M. Maher, R. A. Farber, W. Neuman, W. D. Peterson, Jr., and M. S. Pollack. The HuT series of "carcinogen-transformed" human fibroblast cell lines are derived from the human fibrosarcoma cell line 8387. Carcinogenesis 9, 2073-2079 (1988).

8. H. Palmer, V. M. Maher, and J. J. McCormick. Platelet-derived growth factor or basic fibroblast growth factor induce anchorage independent growth of human fibroblasts. J. Cellular Physiol. 137, 588-592 (1988).

9. P. Hurlin, V. M. Maher, and J. J. McCormick. Malignant transformation of a human fibroblasts caused by expression of transfected T24 HRAS oncogene. Proc. Natl. Acad. Sci. (U.S.A.), 86, 187-191 (1989). (Reprint copy in Appendix.)

10. J. M. Frazier, C. A. Tyson, C. McCarthy, J. J. McCormick, D. Meyers, G. Powis, and L. Ducat. Malignant transformation of human fibroblasts as a carcinogenesis model. Potential use of human tissues for toxicity studies and testing -- a minireview. Toxicity and Appl. Pharmac., 97:387-397, 1989.

11. D. M. Wilson, D. G. Fry, V. M. Maher, and J. J. McCormick. Transformation of diploid human fibroblasts by transfection of N-ras-oncogenes. Carcinogenesis, 10:635-640, 1989.

12. J. J. McCormick and V. M. Maher. Malignant transformation of mammalian cells in culture, including human cells. Environ. Mol. Mutag. (in press, 1989).

13. H. J. Palmer, V. M. Maher, and J. J.McCormick. The effect of retinoids on growth factor-induced anchorage independent growth of human fibroblasts. In Vitro Cellular and Develop. Biol. (in press).

14. D. M. Wilson, D. Yang, J. E. Dillberger, S. E. Dietrich, V. M. Maher, and J. J. McCormick. Malignant transformation of human fibroblasts by a transfected N-ras oncogene. Cancer Res. (submitted).

15. D. G. Fry, L. D. Milam, J. E. Dillberger, V. M. Maher, and J. J. McCormick. Malignant transformation of immortalized human fibroblasts by transfection with v-K-ras. Oncogene (submitted).

B. Monographs, Review Articles, or Chapters in Books.

1. J. J. McCormick, D. G. Fry, and V. M. Maher. Transformation of human fibroblasts by carcinogens or transfection of oncogenes. In: Genetic Toxicology of Environmental Chemicals, Part A: Basic Principles and Mechanisms of Action. C. Ramel, B. Lambert, and J. Magnusson (eds.), Alan R. Liss, New York, NY, pp. 295-303 (1986). (This paper summarizes the results of research conducted on DOE Contract 4659 which preceeded and led to the present DOE grant (ER60524). It is listed here because the work

is directly related to the present research and was published after the preceding Progress Report was submitted in 1988.)

2. V. M. Maher and J. J. McCormick. Role of DNA lesions and repair in the transformation of human cells. In: Mechanisms of Cellular Transformation by Carcinogenic Agents. D. Grunberger and H. Goff (eds.), Pergamon Press, New York, NY, pp. 135-149 (1987).

3. P. Herrlich, M. Imagawa, V. Maher, K. Sato, J. J. McCormick, P. Angel, M. Karin, I. Baumann, C. Luecke-Huhle, and H. J. Rahmsdorf. The molecular basis for the UV response: cis and trans-acting elements responsible for gene induction. In: Accomplishments in Oncology. The Role of DNA Amplification in Carcinogenesis, Vol. 2, Part 1. H. zur Hausen and J. R. Schlehofer (eds.), J. B. Lippincott Co., Philadelphia, PA, pp. 95-105 (1988).

4. J. J. McCormick, D. G. Fry, P. J. Hurlin, T. L. Morgan, D. M. Wilson, and V. M. Maher. Malignant transformation of human fibroblasts by transfected oncogenes. In: Proceedings of the Workshop on Cell Transformation Systems Relevant to Radiation Induced Cancer in Man. IOP Publishing Ltd., Bristol, UK (in press, 1989).

5. J. J. McCormick, D. G. Fry, P. J. Hurlin, T. L. Morgan, and V. M. Maher. Malignant transformation of human fibroblasts by oncogene transfection or carcinogen treatment. In: Fifth International Conference on Environmental Mutagens (ed.) M. Mendelsohn. A. R. Liss, Inc. New York (in press, 1989).

C. Abstracts of Papers Presented at National and International Meetings.

1. D. G. Fry, V. M. Maher, and J. J. McCormick. Characterization of diploid human fibroblasts transformed following transfection with the SSV provirus. J. Cellular Biochemistry, Suppl., 11A, 25, 1987.
2. V. M. Maher, H. Palmer, and J. J. McCormick. Platelet-derived growth factor (PDGF) induces anchorage independent (AI) growth of human fibroblasts (HP). J. Cellular Biochemistry, Suppl., 11A, 34, 1987.
3. J. J. McCormick, R. Schilz, and V. M. Maher. Growth factor requirements of normal and fibrosarcoma-derived human fibroblasts. J. Cellular Biochemistry, Suppl., 11A, 51, 1987.
4. J. J. McCormick, R. Schilz, P. A. Ryan, and V. M. Maher. Growth factor requirements of normal, transformed, and fibrosarcoma-derived human fibroblasts. Proc. Amer. Assoc. Cancer Res., 28, 56, 1987.
5. D. M. Wilson, V. M. Maher, and J. J. McCormick. Transformation of normal diploid human fibroblasts by a transfected N-ras oncogene. Proc. Amer. Assoc. Cancer Res., 28, 152, 1987.
6. P. J. Hurlin, V. M. Maher, and J. J. McCormick. Tumorigenic transformation of human fibroblasts following transfection of the T24 H-ras oncogene. Third Annual Meeting on Oncogenes, Frederick, MD, July, 1987.
7. J. J. McCormick, R. Schilz, P. A. Ryan, and V. M. Maher. Growth factor requirements of normal, transformed, and fibrosarcoma-derived human fibroblasts. FASEB Summer Research Conferences, Copper Mountain, CO, July, 1987.

8. J. J. McCormick, D. G. Fry, P. Hurlin, D. M. Wilson, and V. M. Maher. Malignant transformation of human fibroblasts by oncogene transfer. Carcinogenesis Studies in Human Tissues and Cells. Denmark, October, 1987.

9. R. J. Schilz, V. M. Maher, and J. J. McCormick. Cell lines derived from human fibrosarcomas and human fibroblasts transformed in culture spontaneously or after carcinogen treatment exhibit growth factor independence. J. Cellul. Biochem., 12A, 156, 1988.

10. J. J. McCormick, P. J. Hurlin, D. Wilson, J. Dillberger, D. G. Fry, and V. M. Maher. Malignant transformation of immortalized human fibroblasts by transfection with v-Ki-ras. Proc. Amer. Assoc. Cancer Res., 29, 115, 1988.

11. D. G. Fry, L. Milam, J. Dillberger, V. M. Maher, and J. J. McCormick. Malignant transformation by transfection with v-Ki-ras. Proc. Amer. Assoc. Cancer Res., 29, 115, 1988.

12. J. J. McCormick, D. G. Fry, P. J. Hurlin, D. M. Wilson, and V. M. Maher. Malignant transformation of human fibroblasts by oncogene transfer. International Symposium on DNA Repair, Chromosome Alterations, and Environmental Pollution. Moscow, U.S.S.R., July, 1988.

13. J. J. McCormick, P. J. Hurlin, D. G. Fry, J. E. Dillberger, D. Yang, and V. M. Maher. Malignant transformation of human fibroblasts caused by expression of T24 H-ras oncogene and the K-ras provirus. Gene Regulation and Oncogenes, AACR Special Conference in Cancer Research, Chatham, MA, October, 1988.

14. D. M. Wilson, D. Yang, J. E. Dillberger, V. M. Maher, and J. J. McCormick. Malignant transformation of a human fibroblast cell line by an N-ras oncogene. Gene Regulation and Oncogenes, AACR Special Conference in Cancer Research, Chatham, MA, October, 1988.

15. J. J. McCormick, P. J. Hurlin, D. M. Wilson, D. G. Fry, and V. M. Maher. Malignant transformation of human fibroblasts by transfection of oncogenes. J. Cellular Biology, Suppl., 13B, 35, 1989.

16. J. J. McCormick and V. M. Maher. Malignant transformation of human fibroblasts by transfection of oncogenes. Cell Transformation Systems Relevant to Radiation Induced Cancer in Man, Dublin, Ireland, April 1989.

17. J. Jankun, L. D. Milam, V. M. Maher, and J. J. McCormick. Increased expression of U-plasminogen activator is correlated with oncogene-induced transformation of human fibroblasts to malignancy. Second International Workshop on Molecular and Cellular Biology of Plasminogen Activator. Brookhaven National Laboratory, Long Island, NY, April, 1989.

18. J. J. McCormick, R. J. Schilz, and V. M. Maher. Human fibrosarcoma-derived cells grow without exogenous protein growth factors. Amer. Assoc. Cancer Res., 1989.

19. J. J. McCormick, and V. M. Maher. Malignant transformation of human fibroblasts by oncogene transfection or carcinogens. Fifth International Conference on Environmental Mutagens, 1989.

20. J. M. Scheid, J. J. McCormick, and V. M. Maher. Sequencing polymerase chain reaction amplified ras genes from transformed

human fibroblasts to determine activation. Molecular Biology of Disease. Denver, CO, July 1989.

III. OVERVIEW OF THE STATUS OF THE PROPOSED RESEARCH AT THE BEGINNING OF THE PROJECT - FALL OF 1986 AND THE ORIGINAL OBJECTIVES PROPOSED.

A. Overview.

The overall or broad objective of our studies is to determine the number and kinds of changes human cells must undergo to transform them into malignant cells. Such information on the nature of the steps in the transformation of normal human cells into tumorigenic cells is potentially very valuable since it could lead to determination of the rate-limiting step if such exists, and to the development of strategies to block one or more steps in the process, especially in persons at high risk for tumors. Furthermore, if the individual steps were understood, one could better judge the potential harmfulness of environmental agents as a cancer risk in individuals as well as populations. Such knowledge should also allow one to build statistical models for the risk of cancer based on mechanistic considerations, rather than on tumor frequencies alone. This information would be of special interest to DOE since radiation and chemical carcinogen exposure are hazards in many of the activities of the Department.

In our studies of carcinogen-induced transformation, we use human cells because of our interest in the effects of carcinogens in causing mutations and cancer in human beings. We use fibroblasts as the target cells because this cell type is representative and is especially useful since the techniques for culturing fibroblasts are highly developed which allows one to carry out a wide range of experiments which would not otherwise be possible, e.g., techniques for transfecting genes of interest into such cells and demonstrating expression. Another reason to use fibroblasts as the

target cells is that animal fibroblasts have been widely used in transformation assays, and the results from such studies may serve as a guide to the kinds of changes one might expect to find in human cells, as well as to the kinds of experiments to carry out.

Before outlining the specific objectives of the research it seems important to provide a brief summary of the status of carcinogen-induced malignant transformation of mammalian cells in culture, including human cells. The reviewers may be aware that diploid human cells are reported to be difficult to transform into the malignant state by carcinogens. What may not be as widely recognized is that this is also the case with non-human diploid mammalian cells in culture. The majority of the assays for carcinogen-induced transformation of mammalian cells use highly-selected cell lines which have already acquired an infinite life span in culture, as well as other unrecognized properties that may predispose the cell to transformation into the malignant state. Examples of such widely-used cell lines are the C3H10T1/2 clone 8 cell line of Reznikoff, Brankow, and Heidelberger (1973) and the Balbc/3T3, clone A31-714, cell line developed by Kakunaga (1973). The only assay where one begins with finite life span diploid fibroblasts is that using Syrian hamster embryo cells prepared from pregnant animals. Most commonly the cells are used in a one-step assay where the cells are treated with carcinogen and scored for induced altered colony morphology (DiPaolo et al., 1969). It has been demonstrated (Barrett and Ts'o, 1978) that after extended subculturing (30 to 70 population doublings), malignant cells may arise. This validates the claim that the morphologically-transformed cells are part of a series of changes which can lead to the malignant state. But the original assay itself does not yield malignant cells and what is more, a recent critical review (Jones et al.,

1988) indicates that the altered SHE colony assay itself is not reproducible, even in the hands of highly experienced workers. Nevertheless, the fact that carcinogen-treated diploid Syrian hamster embryo cells can on occasion give rise to malignant cells after extended subculturing makes them different from human cells (see below). However, just as with human fibroblasts there are no methods to reproducibly transform normal fibroblasts into malignant cells by carcinogens.

Although human cells are reported to be difficult to transform, there have been a number of reports of carcinogen-induced malignant transformation of human fibroblasts (cf. **Mutat. Res.**, Vol. 199, No. 2, 1988. Special issue edited by McCormick and Maher). But in spite of serious attempts to repeat these experiments (see below) we never succeeded in obtaining malignant cells, i.e., cells able to grow progressively and invade and erode normal host tissue (see Progress Report, Part IV). We hypothesized that since malignant transformation involves multiple changes within a cell and, therefore, successive clonal selection of cells that have undergone intermediate changes must occur, one possible explanation for the failure to achieve the malignant state is the inability to recognize cells that have undergone intermediate changes so as to expand the population, expose the cells a second time, cause further changes, etc.

Such considerations led us to propose to the DOE in 1986 to transform normal human cells to full malignancy by a sequential transfection of dominant-acting oncogenes derived from acutely transforming RNA tumor viruses and known to be active transforming agents in fibroblasts of some vertebrate species. The assumption underlying these studies was that if a cell's proto-oncogene was activated in the same manner as the transfected oncogene, it would cause the cell to express the same phenotype as observed in the

transfected cell. Oncogenes from DNA tumor viruses have little homology to any genes in the vertebrate genome. Such oncogenes have not been utilized for our studies since there is no evidence that such viruses are causally involved in human fibroblastic tumors in vivo.

The expectation was that by transfection of suitable oncogenes, one could derive a series of cell lines, clonally-derived from each other. The first cell line would be the normal parental cells. The second line would be derived from the first by transfection of an oncogene and would be a step closer to expressing the malignant phenotype. The third cell line would be derived from the second by transfection of another oncogene and it would be still closer to expressing the malignant phenotype, etc. Since human fibroblasts in culture are karyotypically stable, the hope was that each of the cell lines would be genetically stable. The last cell line in the lineage would, of course, be malignantly transformed. Each of the cell lines would be reserved as frozen stocks. If the studies worked as hoped, one would have frozen stocks of human fibroblasts one step removed from malignancy (as well as two steps, etc.). Even more importantly, one would know that the expression of a specific oncogene could malignantly transform a specific cell line. This would allow one to utilize carcinogen treatment to activate the corresponding proto-oncogene. If cells expressing the "correct" phenotype were obtained, one could then determine if the oncogene one expected to be expressed was expressed.

We have successfully carried out these studies and by transfection have obtained malignant transformation. The process involves four sequential clonal selections (see below). Four cell lines, as well as the parental cell line, are available for further study. However, the process of carcinogenesis is much more complicated than the simple model described

above. Our future DOE studies propose to elucidate the process of carcinogen-induced malignant transformation by substituting ionizing radiation for oncogene transfection as described above, to examine various phenomena involved in transformation, as well as various anomalies noted to provide a sufficiently detailed description of the transformation process to develop realistic models.

B. Specific Project Objectives as Originally Proposed.

1. We will test a series of selected "sarcoma-related" oncogenes, i.e., derived from feline sarcoma viruses or isolated from fibrosarcomas and/or known to transform animal fibroblasts, for their ability to transform human fibroblasts in culture and assay the transfectants for "transformation phenotypes", phenotypes of tumor-derived cells, i.e., altered cellular and colony morphology, anchorage independence, focus-formation, growth factor independence, infinite life span, "immortality", and tumorigenicity in athymic mice. Cells that have acquired "transformation phenotypes" will be assayed to see if they possess and express the appropriate oncogene. Using appropriate oncogenes, we will secondarily transfect cells that have already acquired one or more of the "transformation phenotypes" and characterize this second set of transfectants as above.

2. At the same time as the above studies with "sarcoma-related" oncogenes are underway, we will concentrate on transforming human cells to indefinite life span in culture, using oncogenes that are reported to cause cellular "immortality" in various animal cell systems.

3. When and if tumors arise from injection of cells from the above studies, they will be characterized in detail.

IV. MAJOR RESEARCH ACCOMPLISHMENTS

A. Transformation of finite life span human fibroblasts with an H-ras oncogene.

A significant proportion of human tumors from various sites in the body contain activated oncogenes from the *ras* family. This finding, which suggests that the *ras* oncogenes are involved in causing such tumors, has prompted investigations into the role *ras* oncogenes play in bringing about transformation of cells in culture. Transfection studies using primary or early passage rodent fibroblasts as recipients of *ras* oncogenes indicate that these genes act in a dominant manner to cause transformation. The transformed phenotypes observed in *ras* oncogene-transfected rodent fibroblasts include morphological alteration, focus formation, anchorage independence, and, in some instances, tumorigenicity (1-5). Several of these studies indicate that high expression of the transfected *ras* oncogene is required for the induction of the tumorigenic phenotype in such fibroblasts (2-5).

In contrast to the results achieved using rodent fibroblasts, the results of most DNA transfection studies in which early passage diploid human fibroblasts were used as the recipients suggested that these cells are more resistant to transformation by *ras* oncogenes. For example, several groups have reported that following *ras* oncogene transfection, human fibroblasts failed to exhibit morphological transformation (5,6), focus formation (6), anchorage independence (5), or tumorigenicity (5-7). Sager *et al.* (6-8) reported that following transfection of normal human fibroblasts with the *ras* oncogene in a plasmid which contains SV40 transcriptional enhancer sequences, expression of the H-*ras* p21 could

be detected, but this did not result in morphological transformation or focus formation, or tumorigenicity.

We hypothesized that the reason for the negative results with human fibroblasts was that the level of expression of the ras oncogene was not high enough to cause measurable effects. In an attempt to achieve high expression of the T24 H-ras oncogene in early passage, diploid human fibroblasts we, Hurlin et al. (9), used a plasmid (pHO6T1), in which the oncogene is inserted between two sets of transcriptional enhancer sequences, one 5' and the other 3' to the ras oncogene and which carries the neo gene coding for resistance to G418.

Frequency of Transfection to G418 Resistance. Plasmid pHO6T1, which contains the T24 H-ras oncogene, was chosen for transfection of diploid human fibroblasts because of its demonstrated ability to transform early passage rodent fibroblasts into malignant cells (4) and because in our preliminary experiments, other plasmids containing the T24 H-ras oncogene proved to be ineffective in transforming human fibroblasts. Plasmid pHO6N1, which contains the normal, endogenous H-ras gene, and plasmid pHO6 which has no ras gene were used as controls in the transfection experiments. Recombinant plasmids were introduced into early passage diploid human fibroblasts using a modified Polybrene/DMSO transfection technique. To increase the probability that colonies selected for resistance to G418 represented unique transfection events, transfection and selection of cells were performed in the same culture dish. Each plasmid construction yielded approximately the same number of G418 resistant colonies per μg of transfected plasmid, i.e., 200 per 10^6 target cells.

Transformation to Altered Morphology. G418 resistant colonies were microscopically examined for evidence of altered cellular morphology two wks following transfection and selection in G418 medium. Only pHO6T1 induced colonies of morphologically transformed cells which divided rapidly, were highly refractile, and grew in irregular patterns, rather than the highly-oriented pattern of normal human fibroblasts.

Transformation to Focus Formation. Cells transformed following transfection with pHO6T1 could be also identified by their ability to grow into a 3-dimensional array of cells, a focus, on top of a monolayer of contact-inhibited cells. The number of foci observed per dish following three weeks of growth under non-selective conditions was approximately 10-fold greater than the number of morphologically transformed colonies observed after two weeks of G418 selection. No foci were generated following transfection of the control plasmids. Microscopic inspection of the morphology of the cells making up the focus revealed that not all cells capable of forming foci exhibited the transformed morphology. Thus, the number of foci was a more sensitive determination of the frequency of transformed cells following transfection with pHO6T1 than was the number of morphologically transformed colonies.

Induction of Anchorage Independence. Morphologically transformed, G418 resistant colonies obtained following transfection with pHO6T1, and control colonies obtained with the control plasmid, pHO6N1, were compared for ability to form colonies in soft agar. No colonies were observed in the control dishes (frequency, $<1.25 \times 10^{-4}$). In contrast, the oncogene transformed cells showed a frequency of 76×10^{-4}.

Analysis for the Presence of Transfected Sequences. G418-resistant colonies that exhibited the transformed morphologically were isolated, pooled, and expanded for several population doublings. DNA was digested with Xba I and Hind III (double digestion) to liberate specific size fragments diagnostic for an intact region of pHO6T1 containing the T24 H-ras oncogene. Cells exhibiting the transformed morphology contained the diagnostic fragments. Their intensity was approximately half that of the endogenous ras sequences, indicating that the cells contain only one copy of the transfected ras gene.

We found that the majority of the morphologically transformed cells, even if passaged extensively in the presence of selective medium, eventually reverted to a normal morphology. These reverted cells did not form foci or colonies in agar. However, Southern blot analysis showed that the revertants still possessed the transfected oncogene.

Expression of the T24 H-ras Oncogene in Transformed Cells. Seven independent, morphologically transformed clonal populations obtained after pHO6T1 transfection and 5 clonal populations that reverted to the normal fibroblast morphology were analyzed for the presence of the mutated form of p21 by radioimmunoprecipitation analysis using monoclonal antibody. In cells transfected with the control vector and in pHO6T1 transfectants that reverted, the characteristic p21 doublet was expressed, but only the pHO6T1 transfectants exhibiting a transformed morphology expressed an additional p21 species that co-migrated with a p21 species expressed in the human bladder carcinoma cell line T24. The mutant species migrated immediately below the slower migrating, normally present p21 species.

A similar pattern of expression was observed in all of the morphologically transformed populations analyzed. The additional p21 species was not observed in any of the reverted populations, or in control, vector (pHO6) transfected populations tested. RNA analysis of cytoplasmic RNA samples indicated that transfectants maintaining the transformed morphology expressed 4 times higher than normal levels of H-ras specific RNA.

Tumorigenicity. Two G418-resistant, morphologically transformed clonal populations were assayed for tumor-forming ability in athymic mice. No tumors were observed over a period of greater than one year. However, progeny of the morphologically transformed cells had reverted to the normal fibroblast morphology during the expansion required to obtained enough cells for this assay. When these revertant cells were continuously passaged as part of a life-span assay, they senesced at a population doubling level comparable to that of normal fibroblast populations.

Conclusions. These results show that normal diploid human fibroblasts can be transformed into morphologically altered, focus forming, anchorage independent cells following transfection of the T24 H-ras oncogene, but the cells do not acquire an infinite life-span and are not tumorigenic. These positive results were generated using a plasmid specifically designed to elicit high expression of the T24 H-ras oncogene in mammalian cells by inserting the oncogene between two sets of transcriptional enhancer sequences. Therefore, we, Hurlin et al. (9), interpret our results to indicate that a sufficiently high level of expression of the T24 H-ras oncogene was achieved to cause the transformation of normal human fibroblasts, and that the pSV2-T24 plasmid of Sager et al. (8) was not capable of generating such levels. This interpretation does not explain

the negative results of Spandidos (5), who reported that transfection of normal human fibroblasts with pHO6T1 did not cause anchorage independence or any obvious morphological transformation. However, Spandidos used a different method of transfection (calcium phosphate) then we did (DMSO/Polybrene), and examined a relatively small number of independent G418 resistant colonies ($\sim$200) compared to the total number we examined (>2461).

The results of numerous ras oncogene transfection studies carried out with rodent fibroblasts suggest that high expression of the T24 H-ras oncogene can be attained, and that with such expression the tumorigenic phenotype is induced (2-5). Our studies with human fibroblasts indicate that the transfected oncogene is expressed at low levels, compared to what has been observed in rodent fibroblasts, and that such expression is lost in most cells as they are passaged. They show that it is possible by transfection of an oncogene to cause these finite life span human cells to acquire many of the characteristics of tumor-derived cells, but, not to acquire an infinite life span or to become malignantly transformed.

B. Transformation of finite life span human fibroblasts with N-ras oncogenes.

To determine if an N-ras oncogene could cause transformation of normal diploid fibroblasts, we, Wilson et al. (10), transfected several early-passage foreskin-derived cell lines with plasmids containing an activated (mutated) N-ras gene. One of them, pSV N-ras, is a high expression plasmid which contains two Maloney leukemia virus long terminal repeats (LTR's). The cellular promoter of the N-ras oncogene from human leukemia cell line 8402 was eliminated and the gene inserted between an LTR and the neo gene coding for Geneticin resistance (11) so that transcription initiated at the first LTR must transcribe the N-ras gene before transcribing the neo gene. With this construction, the likelihood that cells selected for Geneticin resistance will also express the N-ras oncogene is very high. In the other plasmid, pNR-MG1, transcription of the N-ras oncogene from human fibrosarcoma cell line HT1080, is driven from its endogenous promoter and the neo gene is transcribed from an SV40 viral promoter (5). As a negative control, the cells were transfected with pSV2neo which lacks an oncogene.

The transfected populations were selected for resistance to Geneticin and the drug-resistant colonies were examined for evidence of morphological transformation. The frequency of transformation to Geneticin resistance in a series of experiments averaged 2 to 3 x 10^{-5} with pSV N-ras, and 1 to 3 x 10^{-4} for pNR-MG1 and pSV2neo. Of the pSV N-ras transfectants that expressed the neo gene and formed drug-resistant colonies, a high proportion ($\geq$70%) were composed of cells with highly altered morphologies. The cells were highly refractile, vacuolated, of highly irregular shape, and did not exhibit the oriented growth pattern characteristic of normal human fibroblasts. Some were multi-nucleated. Less than 5% of the Geneticin-resistant colonies

formed after transfection with pNR-MG1 contained morphologically-altered cells and the morphological changes were very much less distinct than in the pSV N-ras transfectants. No colonies containing morphologically altered cells were found with the control plasmid.

The N-ras transfected populations were also assayed for the ability to form foci on a confluent monolayer. The cells were not selected in Geneticin, but allowed to grow to confluence. Within two weeks after transfection with pSV N-ras, very distinct, dense foci, composed of highly refractile, irregularly shaped cells, could be observed on a monolayer of contact-inhibited fibroblasts. By day 10 after transfection with pNR-MG1, less distinct foci could also be seen on a confluent background but those foci were difficult to recognize and later the areas became indistinguishable from the background.

Stability of the morphologically-altered phenotype. 80 Geneticin-resistant colonies or foci composed of morphologically transformed cells obtained after transfection with pSV N-ras and 20 colonies or foci obtained after transfection of pNR-MG1 were isolated and cultured, along with several age-matched Geneticin-resistant colonies composed of cells with normal morphology obtained from pSVneo transfection. At the time these cells were isolated, they had undergone the equivalent of 20 to 25 population doublings (cell generations) since their parental cell lines were initiated from foreskin material. Cells derived from pNR-MG1 transfection reverted to a normal morphology within 5 to 10 population doublings after being isolated, but exhibited the same life span in culture as their age-matched pSV2neo-transfectant controls. Cells derived from most of the colonies or foci obtained with pSV N-ras either reverted to a normal morphology after only 5 to 10 cell generations or senesced at that time. However, progeny cells from

two of the latter colonies (designated N-ras cell strain 1 and 2) did not senesce prematurely or revert, but maintained their transformed morphology stably for 15 to 20 more cell generations. These cell strains and two normal age-matched control cell strains derived from pSV2neo were studied further.

Focus-formation, anchorage independence and expression of N-ras p21. N-ras cell strain 1 was assayed and found to form foci on a background of normal cells. No such foci were observed in the dishes seeded only with control cells. N-ras cell strain 1 and an age-matched pSV2neo-derived control cell strain were also assayed for anchorage independence. The N-ras-transformed cell strain formed colonies in agar at a frequency 80-fold higher than the control. The transformed cell strains were shown to express higher than normal amounts of N-ras oncogene-encoded protein, using v-H-ras (Ab-1) which in human cells will precipitate H-ras, K-ras, and N-ras p21s, and v-H-ras (Ab-2) which will precipitate human H-ras and K-ras p21s, but not human N-ras p21.

Other parameters related to transformation. The life span in culture of the two N-ras strains did not differ from that of the age-matched pSV2neo-derived control cell strains, i.e., 40 to 45 total cell generations since initiation. Injection of X-irradiated athymic mice with 1-2 x 10^6 cells from N-ras-transformed cell strain 1 or 2 along with 9 x 10^6 control cells did not yield any tumors. The injected animals were examined for tumors for more than nine months. When 10^6 human fibrosarcoma-derived cells are injected, tumors routinely develop in less than 6 wk.

Conclusions. These results indicated that early passage human fibroblasts can be transformed into morphologically-altered, focus forming cells by transfection with human N-ras oncogenes. The degree of morphological alterations observed was much higher in cells transfected with

a plasmid designed to give high expression of the N-ras oncogene from a viral LTR (pSV N-ras) than in those that received a plasmid in which the N-ras oncogene was transcribed from its endogenous promoter (pNR-MG1). Marshall et al. (12) failed to observe transformation of diploid human fibroblasts following transfection of the N-ras oncogene from HT1080 cells inserted into the plasmid pSV2neo, in which transcription of the N-ras oncogene is initiated from an SV40 promoter. These results suggest that to see the altered morphology, the level of expression of the transfected ras oncogene has to be high.

We do not know what caused the cells to revert or to enter crisis relatively soon after isolation. If reverting to a normal morphology was correlated with a decrease in the level of expression of N-ras, as was reported for H-ras by Hurlin et al. (9), this might have been the result of down regulation (13) by overmethylation of the gene (14). Premature senescence may have resulted from overexpression of the transfected oncogene, since this is what was seen with primary rat embryo fibroblasts transfected with cloned ras oncogenes (12) and Franza et al. (15) reported that transfection of baby rat kidney cells with highly expressed ras oncogenes almost invariably resulted in premature senescence of the cells isolated from the transformed colonies.

The results showing that transfection of an N-ras oncogene, even one cloned into a high expression plasmid, was insufficient to transform early-passage diploid human fibroblasts into tumorigenic cells supports the hypothesis that carcinogenesis is a multi-step process. This lack of tumorigenicity is consistent with the findings of Weinberg and his colleagues (3) and others (16-18) who showed that transfection of a second oncogene,

such as myc, was needed if finite life span rodent cells were to be malignantly transformed by transfection of ras oncogenes.

C. Studies on the relationship between parental cell lines and human fibroblasts reported to have been transformed into the malignant state spontaneously or following carcinogen treatment.

Kakunaga (19,20) reported that following a single exposure of skin fibroblasts derived from the lip of a normal person (KD) to 4-nitroquinoline-1-oxide or N-methyl-N'-nitro-N-nitrosoguanidine and extensive subculturing of the progeny of the surviving target cells, foci were observed developing on top of confluent monolayers of the progeny of the target cells. Cells isolated from these foci were propagated further and found to be malignant, i.e., to form progressively-growing invasive sarcomas in athymic mice. Kakunaga later designated these cell lines HuT-11 cells, e.g. HuT-12, HuT-13, and HuT-14. McCormick and Maher and their colleagues tried unsucessfully to confirm the results of such experiments, using the same target cells (KD) and the same carcinogens. They sometimes induced foci-formation in the human fibroblasts, but failed to generate malignant cells by such treatment. This failure may have resulted from some critical difference between the procedures we used and those of Kakunaga. However, in 1987, ten years after the report of Kakunaga's successful study, McCormick and Maher were asked to edit a special issue of **Mutation Research** (Vol. 199, No. 2, 1988) devoted to in vitro transformation of human cells in order to clarify some of the controversies that had arisen during that 10 year period. In connection with that assignment, they compared the characteristics of Kakunaga's HuT cell strains, as well as several other human fibroblast cell lines which had been reported to have spontaneously transformed into malignant cells (Thielmann et al., 10a; Mukherji et al., 20b) and the corresponding parental cell lines

from which they arose (21). The malignant cell lines and their reputed parental cell lines were tested to see if they shared common enzyme patterns, HLA antigens, restriction fragment length polymorphisms, and marker chromosomes. To our surprise, each of the tests showed marked differences between the KD cells and the HuT cells, even though each of the different HuT cell lines exhibited identical patterns in each test. This was also the case with Thielmann's cells. Mukherji's cells grew so poorly they could not be checked for HLA and isozyme patterns (21). We concluded from that study that unless Kakunaga had actually used some normal cell line other than KD as his target cells, there apparently was no example of malignant transformation of human fibroblasts in culture.

What remained unclear was whether Kakunaga had begun with non-malignant cells from some other unidentified normal individual and had transformed them to malignancy by the process he described, or whether the "transformation" was a result of contamination of the cells in his experiment with malignant cells derived from a human fibrosarcoma or sarcoma. Since the fibroblast cell lines initiated at that time in Dr. Kakunaga's laboratory from other apparently normal individuals are no longer available (personal communication, T. Kakunaga, 1987), there seemed no direct way to answer this important question. However, late in 1987, McCormick discovered a 1981 paper (22) in which Fogh and his associates reported the isozyme patterns of 100 human cell lines, including the human fibrosarcoma-derived cell line 8387, and he recognized that the pattern of the day 8387 cells matched the pattern McCormick and Maher had recently found for the HuT cell lines (21). The 8387 cell line was established in 1966 by Dr. Paul Price, then of Microbiological Associates, Bethesda, MD, and was first described by Aaronson et al. (23) of the NCI in a paper published in 1970 by scientists from these two institutes.

Since Kakunaga was working at the National Cancer Institute (U.S.A.) in the middle 1970's and it was there that he carried out the studies on the transformation of human fibroblasts, it was at least possible that 8387 cells had found their way into his cultures.

Because the chance of cell lines from two unrelated individuals in the U.S.A. population possessing this very same set of isozyme patterns is less than 4%, we were concerned that the 8387 cell line might have been responsible for the "transformation" observed by Kakunaga and, therefore, we obtained 8387 cells and carried out a series of assays to determine the degree of identity between them and the HuT cell lines. Our results show there were no significant differences between the HuT cells and the 8387 cells. Since as far as is known, the tests we used measure independently-inherited characteristics and since the frequency of the various alleles in the U.S. population is known, one can calculate the odds that, by chance, Kakunaga began with cells from a normal donor (not KD) who possessed characteristics identical to those of the patient whose tumor gave rise to the 8387 cell line. The results showed that the chances are 1×10^{-8}. Additional data for which allelic frequencies cannot be calculated make this even more unlikely. While the isozyme, HLA and RFLP pattern studies were being carried out, photographs of chromosome preparations made earlier of metaphases from 8387, HuT-11, HuT-12, and HuT-14 cells were examined and similarities were observed in the marker chromosomes of the four cell lines. Therefore, a blind study was carried out on three of the cell lines (8387, HuT-12, and HuT-14) by a cytogeneticist who was unaware of the reason for this study or the identify of the cell lines. He found that HuT-12, HuT-14, and 8387 cells had six marker chromosomes in common. When injected subcutaneously into athymic mice, HuT-14 and 8387 cells both produced

undifferentiated sarcomas. The tumors from both cell lines arose after virtually the same latency period, appeared to be identical and had no unusual histological features. Taken all together, these results indicated that the HuT cell lines are in fact derived from the human fibrosarcoma cell line 8387, rather than being the result of malignant transformation of normal fibroblasts by carcinogen treatment. This is consistent with the conclusion that no one has successfully transformed human fibroblasts (21) or human epithelial cells (25) to the malignant state by carcinogen treatment. Clearly, future studies which report human cell transformation must provide evidence that indicates that the transformed cells are a derivative of the parent cell used.

D. Developing an infinite life span diploid human fibroblast cell strain

The results of Hurlin et al. (9) and Wilson et al. (10) with finite life span cells, confirmed the earlier results of Fry et al. (26) who transformed diploid human fibroblasts with a v-sis oncogene and obtained cells able to form very distinct foci and grow in the absence of exogenous growth factors, but which did not acquire an infinite life span and could not form tumors. The results indicated that even if cells are able to exhibit multiple phenotypes characteristic of tumor-derived cells, they still are not capable of forming progressively-growing tumors in athmyic mice. To us, this emphasized the need to obtain cells which had somehow acquired an infinite life span in culture. This is because we reasoned that if the transfected cell was nearing the end of its finite life span it would not be capable of sufficient cell doublings to give a tumor. What was needed was an infinite life span fibroblast strain as recipient for transfection of various oncogenes. The ideal recipient cell strain would be diploid human fibroblasts that exhibited an infinite life span, but otherwise had a

completely normal phenotype. However, human fibroblast cell strains with infinite life spans are very rare, and those that exist exhibit many characteristics of tumor-derived transformed cells. This makes them poorly suited for studies designed to determine the essential changes involved in malignant transformation. For example, human cells "immortalized" following infection with the SV40 virus exhibit morphological transformation, the ability to form colonies in soft agar, chromosomal abnormalities, etc. The two infinite life span human fibroblast cell strains generated by Namba et al. (27,28) by repeated exposure of populations to cobalt-60 (KMST-6) or 4-nitroquinoline-1-oxide (SUSM-1), also exhibit the characteristics found in SV40-immortalized human cells. Therefore, we and our colleagues tried to develop an infinite life span human fibroblast cell strain that had undergone only minimal changes, using transfection of appropriate oncogenes.

We were guided in this attempt by the work of Weinberg and his colleagues (1) who showed that transfection of rat embryo fibroblasts with the v-myc oncogene increased the frequency at which the cells developed into infinite life span cell strains. Using the DMSO/Polybrene method of Morgan et al. (29), we transfected early passage, foreskin-derived normal human fibroblasts with a plasmid carrying the neo gene and a v-myc gene. The transfectants were selected for Geneticin resistance and clonally-derived cell strains were isolated and propagated for many generations. Eventually all cell strains senesced, but one cell strain survived senescence and gave rise to an infinite life span cell strain designated MSU-1.0.

These cells are unique in that they are diploid, non-tumorigenic, morphologically normal, human fibroblasts with normal growth control. These cells are the perfect substrate for further studies on cell transformation since other than the infinite life span phenotype, they have no abnormalities

we are aware of. The development of this cell line was the critical breakthrough in carrying out our transformation studies. Without the infinite life span phenotype (or at least a greatly extended life span), human fibroblasts have too short a life in vitro to acquire by clonal selection all the characteristics necessary for full malignancy. The MSU-1.0 cell line has grown more than 200 population doublings since crisis.

To emphasize the uniqueness of this cell line, one must realize that human fibroblasts have <u>never</u> been seen to spontaneously generate infinite life span in culture. The only workers to successfully generate infinite life span human fibroblasts did so by repeated exposure to 4-NQO or ionizing radiation carcinogen/mutagen treatment (27,28) and they succeeded only twice out of hundreds of attempts. Unfortunately the two cell lines they generated, SUSM-1 and KMST-6, have also acquired many other changes (aneuploidy, morphological transformation, etc.) and so are not very useful for a step-wise analysis of the transformation process.

One can sometimes create infinite life span human fibroblasts by transfection or infection with SV40. However, these cells are also transformed, that is they exhibit anchorage independent growth, morphologically transformed and are aneuploid. In this case, the infinite life span phenotype is a rare secondary affect of SV40 because the vast majority of cells go into crisis and die. However, since the infinite life span cells express multiple transformed characteristics, they too are not very useful for our studies.

From the MSU-1.0 cell line, a spontaneously occurring growth factor variant named MSU-1.1 arose. This cell line has two marker chromosomes. One was made by the fusion of one copy each of chromosomes 12 and 15. The other was made by the fusion of one copy of chromosome 11 fragment apparently arose

with the parm of chromosome 1. There are two normal copies of chromosome 1. This cell line has a shorter doubling time than MSU-1.0 cells and clones better. It has grown more than 200 doublings since crisis.

E. Malignant transformation of the infinite life span human cell strain MSU-1.1 by transfection of an H-ras oncogene

The infinite life span MSU-1.1 cells were then used as the recipient cells for transfection with plasmids containing various ras oncogenes. MSU-1.1 cells have a normal morphology and have exhibited the same near-diploid karyotype for more than 200 population doublings post-crisis. They do not form foci, or produce colonies in soft agar, and they are non-tumorigenic. We, Hurlin et al. (30) made use of this cell strain to determine if transformation of the T24 H-ras oncogene carried in the pHO6T1 plasmid into human cells with an infinite life span not only causes them to exhibit the several characteristics previously seen with finite life span fibroblast cell lines (9), but also the ability to form rapidly growing malignant fibrosarcomas in athymic mice. Because the recipient cells already were expressing the neo gene, the transfectants were identified by their ability to form foci of morphologically transformed cells on a background monolayer of fibroblastic cells.

Cells that had been transfected with pHO6T1 formed distinct foci of morphologically altered cells at a frequency of 10 per 10^6 cells transfected. No foci were observed following transfection with control plasmids containing either the normal human H-ras gene (pHO6N1), or lacking a ras gene (pHO6). Transfection of 5×10^6 MSU-1.1 cells with plasmids containing the T24 H-ras oncogene, but no enhancer sequences (pT24), or with only one SV40 transcriptional enhancer (pSV2-T24) did not induce focus-formation. Six independent populations of morphologically transformed cells (designated

MSU-1.1-T24 strains 1-6) were isolated. They have maintained their transformed morphology for more than 80 population doublings to date. This stability contrasts with what Hurlin et al. (9) found with finite lifespan human fibroblasts transformed following transfection of the T24 H-ras oncogene in pHO6T1.

Growth of MSU-1.1-T24 fibroblasts in serum-free medium and anchorage independence. Schilz et. al. (31) found earlier that human fibrosarcoma-derived cell lines, but not normal human fibroblasts, were capable of proliferating in McM medium (32) containing 0.1 mM calcium instead of the usual 1 mM, and supplemented with the serum replacements of Ryan et al. (32), but lacking epidermal growth factor. When MSU-1.1-T24 cell strains, the parental MSU-1.1 cells, and LG1 cells were compared for ability to proliferate in this medium, the MSU-1.1-T24 cell strains proliferated to numbers 6- to 7-fold higher than the MSU-1.1 cells did, reaching densities equal to that attained in medium containing 10% fetal bovine serum. As expected, LG1 cells could not proliferate. Tests for ability to form colonies in agar showed that the six MSU-1.1-T24 cell strains did so with efficiencies from 3.5% to 23%. There was variation in the size range of colonies detected. For example, the strain that exhibited an agar cloning efficiency of only 3.5%, gave rise to the highest proportion of very large colonies (i.e., having diameters of >300um).

Tumorigenicity of MSU-1.1-T24 cell strains. Four of the six MSU-1.1-T24 cell strains injected into athymic mice formed progressively growing tumors that reached 1 cm in diameter within 30 to 36 days. These were diagnosed as poorly-differentiated, invasive fibrosarcomas with a high mitotic index and a moderate degree of anaplasia. A low proportion of giant multinucleated cells were observed in several of the tumors. Analysis of G-

banded and conventionally-stained chromosomes from MSU-1.1-T24 cell strains, from cells taken from malignant tumors derived from them and from parental MSU-1.1 cell strain showed that the karyotype of each set was identical.

Presence and expression of the T24 H-ras oncogenes. Following digestion with XbaI and HindIII, genomic DNA from each of the MSU-1.1-T24 cell strains was found to contain H-ras hybridizing fragments close to the sizes diagnostic for pHOT1 sequences that include the T24 oncogene. No such sequences were seen in parental MSU-1.1 cells or in LG1 cells. Radioimmunoprecipitation analysis using the ras p21 specific monoclonal antibody Y13-259, indicated that each of them overexpressed a 21 kd protein that migrated to the same position as the overexpressed T24 H-ras oncogene-encoded p21 of the bladder carcinoma cell line. The radiolabeled p21 bands were cut out of duplicate gels and quantitated by scintillation counting. The level of total ras p21 expression in the MSU-1.1-T24 cell strains was 3 to 5 times that expressed in parental MSU-1.1 cells, or in LG1 cells, and was only slightly elevated over that observed in bladder carcinoma-derived cells. Radioimmunoprecipitaion analysis of ras p21 was also carried out on fibroblasts taken from the malignant tumors derived from two of the MSU-1.1-T24 cell strains. The T24 H-ras p21 levels in these cells were comparable to that in their respective focus-derived cell strains, indicate that in vivo selection of cells expressing higher (or lower) levels of the transfected oncogene did not occur.

Transformation studies with other infinite lifespan human fibroblast cell lines. Three other infinite lifespan cell lines which had not been transfected with the v-myc oncogene, also were transfected with the T24 H-ras (pHO6T1) or with the normal H-ras gene (pHO6N1) to determine if expression of the T24 H-ras oncogene could also cause malignant

transformation of such cells. The transfectants, i.e., the SUSM-1 (27) and KMST-6 (28) cell lines of Namba, and an infinite life span cell line generated following SV40 virus infection (GM0637B), were selected for G418 resistance, rather than for focus formation, because unlike the parental MSU-1.1 cell line, these target cell already exhibit a transformed morphology and aberrant growth behavior. The G418-resistant colonies obtained with each cell line were pooled, propagated, and tested for tumorigenicity. Four out of five mice injected with pHO6T1-transfected KMST-6 cells and three out of six injected with pHO6T1-transfected GM0637B cells developed progressively growing malignant fibrosarcomas. No tumors were formed in 5 mice injected with pHO6T1-transfected, SUSM-1 cells generated in two separate experiments, or with any of the pHO6N1-transfected, G418-resistant controls. Immunoprecipitation studies with these pooled populations indicated that the KMST-6 and GM0637B transfectants expressed the T24 H-ras oncogene, but the SUSM-1 transfectants, which failed to produce tumors, did not. In contrast to the 30 to 36 d required for the tumors formed by the focus-derived MSU-1.1-T24 cell strains to reach a diameter of 1 cm, the tumors by the these populations of G418-resistant colonies required 4 to 5 mo to reach this size.

Conclusions. From the results of this study, we concluded that "immortalized", non-tumorigenic human fibroblasts could be transformed into malignant cells by transfection of the T24 H-ras oncogene and its subsequent expression. The tumors formed by the MSU-1.1-T24 cell strains grew progressively and were diagnosed as invasive fibrosarcomas. Since the karyotype of the cells derived from the tumors matched the karyotype of the MSU-1.1-T24 cells injected and both karyotypes were identical to that observed in the non-tumorigenic parental MSU-1.1 cell line from the earliest passage tested post-crisis, we conclude that the tumorigenic phenotype in

these T24-transformed cell strains did not arise as the result of detectable chromosome changes.

It has been proposed from studies with rat embryo fibroblasts that the v-myc oncogene cooperates with a ras oncogene to cause cells to become malignantly transformed. Our studies do not disagree with this hypothesis, but the fact that transfection and subsequent expression of T24 H-ras also induced the malignant transformation of two non-v-myc-transfected infinite lifespan cell lines, suggests that the infinite lifespan phenotype, rather than v-myc expression, is the characteristic of MSU-1.1 cells that complemented ras oncogene expression and allowed the cells to be malignant.

The significance of these in vitro studies of the transformation of human fibroblasts by transfection of activated ras genes ultimately depends on whether activated ras genes play a causal role in the malignant transformation of human fibroblasts in vivo. Recent evidence (33) for the role of activated ras oncogenes in the etiology of colon cancer support this hypothesis. In addition, activated (mutated) N-ras genes have been found in human fibrosarcoma cell lines HT1080 (34) and SHAC (35).

F. Malignant transformation of the infinite life span human cell strain MSU-1.1 by transfection of an N-ras oncogene.

To increase the chances of obtaining MSU-1.1 transfectants which express the oncogene, we (Wilson et al., 36) transfected the N-ras oncogene from human lymphoma cell line 8402 in the high expression plasmid pSV N-ras which contains two Maloney leukemia virus long terminal repeats (LTRs). The endogenous promoter of the N-ras oncogene has been eliminated and the gene has been inserted between an LTR and the neo gene coding for Geneticin resistance so that transcription of both genes is initiated from the LTR. The transfected population was allowed to grow to confluence and examined for

the formation of foci. As a control, MSU-1.1 cells were transfected with pSV2neo which lacks an oncogene. In each of the ten dishes of cells that had been transfected with pSV N-ras, 8 to 15 distinct foci could be seen growing on top of a confluent monolayer after 1 wk. The morphology of the cells was highly anaplastic, with many being epithelioid, refractile and irregularly shaped. Eight foci were isolated and propagated, each from an independent dish, and were designated MSU-1.1-N-ras cell strains 1 through 8. Four of these were chosen for in-depth characterization.

Anchorage independence and growth factor independence of MSU-1.1-N-ras cell strains. The focus-derived cells were tested for ability to grow in soft agar, using conditions which we determined would allow non-transfected MSU-1.1 cells to form less than 2 colonies per dish of 5×10^6 cells and found to form colonies in agar at frequencies reaching 200 to 500 times background. The strains were also tested for their ability to replicate in 0.1 M Ca^{++} McM medium containing 0.1% fetal bovine serum. The parental MSU-1.1 cells replicated only minimally under these conditions, whereas the MSU-1.1-N-ras cells doubled every 24 h, i.e., at the same rate as the parental MSU-1.1 cell line did in the presence of 10% FCS. These focus-derived cells were not stimulated to replicate faster by addition of exogenous EGF, PDGF, or bFGF, whereas their parental cell line responded to such growth factors by an increased rate of replication.

Tumorigenicity of the MSU-1.1-N-ras transformed cell strains. Four strains were injected subcutaneously into mice to examine their tumorigenic potential. All four cell strains gave rise to malignant tumors after a relatively short latency period which were invasive and grew progressively. To determine their metastatic potential, five mice were injected intravenously with cells derived from a sarcoma formed by N-ras cell strain

3. Four mice developed sarcomas in their rear legs and abdominal wall, and the fifth mouse in its dorsal thoracic cavity, but not involving the lungs. Mice injected intracardially with N-ras cell strain 3 and with cells derived from tumors formed from them produced sarcomas in various organs, indicating that the malignant cells had the capacity to form metastatic lesions throughout the body. The level of expression was determined using immunoprecipitation. Each N-ras cell strain, as well as cells derived from tumors formed by them, exhibited overexpression of ras p21 compared to the level found in MSU-1.1 cells, LG1 cells or human fibrosarcoma-derived cell line, 1080, which is known to have activated N-ras oncogene mutated at codon 61 (37). Southern blot hybridization analyses, using N-ras specific sequences showed that each of the transformed cell strains contained additional N-ras specific bands, not present in the MSU-1.1 cells. The patterns seen in cells taken from tumors formed by subcutaneous injection of N-ras cell strain 2 or 3 matched those of their corresponding focus-derived cell strain.

Conclusions. Our data and those of Hurlin et al. (9) argue that overexpression is required for infinite life span human cells to be transformed to the malignant state by transfection of a ras oncogene. Although the HT1080 cells do not exhibit such overexpression, they produce their own platelet-derived growth factor (37). The presence of a mutated N-ras oncogene (34) and the ability to make PDGF may be enough to cause these HT1080 cells to drive their replication. In cells such as MSU-1.1 cells, which do not produce their own PDGF, overexpression of ras p21 may be required in order to accomplish this same driving of replication.

G. Malignant transformation by transfection of human cells with a viral K-ras oncogene.

Fry et al. (38) transfected these same MSU-1.1 cells with plasmid pK4e, which contains the provirus of Kirsten MSV inserted into pBR322. They found that it caused them to develop foci of densely growing, morphologically transformed cells at a frequency of 5×10^{-7}. No foci were observed in cell cultures transfected with pSV2gpt, a control plasmid which does not carry an oncogene. When duplicates of these pSV2gpt-transfected cultures were selected for mycophenolic acid resistance, drug-resistant colonies were detected at a frequency of 1×10^{-4}, indicating that the cells could readily take up and integrate plasmid DNA. Cells from two foci from separate cultures of pK4e-transfected cells were isolated and the populations expanded for further study. Both of these focus-derived cell strains were much less fibroblastic in appearance and formed a less uniform monolayer cells than the parental MSU-1.1 cells. Both cell strains exhibited a significantly increased frequency of colony-formation in semi-solid medium compared to the parental MSU-1.1 cells. Several independent agar colonies were isolated, and the cells were returned to culture to be tested for tumorigenicity.

Tumorigenicity. The tumorigenicity of the two focus-derived cell strains and a population of anchorage independent cells derived from each of them was assayed by subcutaneous injection into athymic mice. All four cell strains produced tumors. In no case were tumors detected in mice injected with the parental MSU-1.1 cells, even eight months following injection. The time required for the tumors to reach 1 cm in diameter ranged from 3 to 10 wk. Except for one tumor which was diagnosed as a fibroma, all the tumors were well-differentiated, invasive sarcomas. Two cell lines produced tumors with abundant matrix that were classified as myxoid sarcomas. The third produced tumors with scant matrix that were classified as spindle cell

sarcomas. Cells derived from portions of four tumors returned to cell culture for further characterization were found to be karyotypically identical to the parental MSU-1.1 cell line. Cells from one of the myxoid sarcomas which developed after subcutaneous injection of cells derived from an agar colony were injected subcutaneously, or into the tail vein to determine their ability to colonize various organs. Cells injected subcutaneously produced myxoid sarcomas at the site of injection; those injected intraperitoneally produced progressively growing, invasive myxoid sarcomas on the peritoneum and on organs in the abdominal cavity; those injected into the tail vein formed invasive spindle cell sarcomas. Fibrosarcomas were found in the lung, lumbar region, abdomen, kidneys, pancreas, and in the rear leg.

Presence and expression of the v-K-ras oncogene. Southern blot analysis of DNA isolated from the two focus-derived cell strains and two tumor-derived strains exhibited distinct patterns of bands that hybridized to the v-Ki-ras probe, bands not present in human placental control DNA. Northern blot analysis demonstrated that the transfected v-K-ras gene was expressed but not overexpressed in the transformed cells.

Conclusions. The results of this study with transfection of a K-ras oncogene is consistent with the results of Hurlin et al. (9) and Wilson et al. (10). The fact that malignant transformation was found with all three ras oncogenes, indicates that the results reported here are not limited to the v-K-ras gene, nor are they likely to be the result of other retroviral sequences present in the pK4e plasmid utilized for the present experiments. The ability to reproducibly generate malignantly transformed human fibroblasts may allow development of techniques to examine the induction of this phenotype by exposure of cells to radiation or chemical carcinogens and

facilitate studies of the changes involved. It can also provide a useful model to examine directly the role of such activated ras genes in human cell transformation.

We and our colleagues have shown that the MSU-1.1 cells can be malignantly transformed by transfection of the H-ras (30) or N-ras (36) oncogene when the oncogene is driven by a promoter that causes overexpression of the mutant ras protein, and that no such transformation occurs if the oncogenes are carried in plasmids engineered to give a normal level of expression of the p21s. Yet, Fry et al. (38) succeeded in causing the malignant transformation of the MSU-1.1 cells by transfection of the v-K-ras provirus, even though in the case of K-ras-malignantly transformed cells, the p21 of the mutant K-ras is not overexpressed. This may reflect the fact that the K-ras oncogene differs from the H-ras or N-ras oncogenes in that it carries two activating mutations. Our interpretation of these data is that the MSU-1.1 cell line is two steps away from malignant transformation since normal levels of expression of the activated H-ras or N-ras oncogene do not malignantly transform these cells. Considered from the point of view of the DNA changes in the proto-oncogenes that would be required for MSU-1.1 cells to become malignant, it appears that the cell must undergo a base pair change in codon 12, 13, or 61 of a ras gene and this ras gene must be rearranged so it is under the control of a strong promoter. Consistent with this interpretation is the fact that recently two spontaneous transformants have arisen in the MSU-1.1 cell line after extended subculturing. These two transformants, which are malignant, must have arisen independently since each has unique marker chromosomes. In contrast, spontaneous transformants have never been seen in the cell line strain that represent an earlier stage in this lineage (e.g., MSU-1.0) which is consistent with this interpretation.

We are in the process of determining whether the ras genes of the two spontaneous MSU-1.1 malignant transformants have activating mutations.

H. Studies on the modulation of activated ras expression.

As noted above Wilson et al. (36) and Hurlin et al. (30) showed that overexpression of a mutant H- or N-ras oncogene in MSU-1.1 cells causes them to develop to foci and these focus-derived cells are malignantly transformed. But Schilz et al. (31) found that human fibrosarcoma-derived cells do not overexpress ras, even though some of these cell strains have a mutated ras. When Hurlin et al. (9) used a plasmid in which the promoter on the H-ras oncogene resulted in normal levels of expression of p21, normal diploid cells did not become morphologically transformed. Therefore, it was of interest to determine if the effects observed in the infinite life span were directly related the level of expression of the transfected ras oncogene. One way to do this was to transfect MSU-1.1 cells with a plasmid in which the T24 H-ras oncogene was activated by the metallothionein promoter (MT-ras).

Reynolds et al. (39) reported that Rat-1 cells transfected with the MT-ras gene changed from a normal to a transformed morphology and the morphology was even more altered in the presence of zinc. The difference in the level of ras-specific RNA between control cells exposed to 100 uM zinc and transfected cells exposed to 100 uM zinc was 500-fold. The difference in levels of ras-specific RNA between transfected cells exposed to zinc and those not exposed to zinc was about 30-fold. These results and other data indicated that the concentration of zinc in the medium could effectively modulate the expression of the mutant H-ras gene.

We have transfected the MSU-1.1 cell strain with the MT-ras plasmid. Foci were seen when the cells were treated with 150 uM Zn^{++}. Clonal populations isolated from the foci showed normal morphology when exposed to

medium not containing Zn^{++} and a progressively more transformed morphology when the Zn^{++} concentration was increased stepwise up to 150 uM. Studies on the immunoprecipitation of the H-*ras* protein when cells are induced with Zn^{++} or not induced are underway.

I. Correlation between levels of protease activity (plasminogen activator) and degree of oncogene induced transformation of human fibroblasts.

Denhardt and colleagues (40) reported that cathepsin L is the major protein excreted into medium by NIH3T3 cells transfected with the activated H-ras gene, but not by the parent 3T3 cells. Many other workers have shown that proteases, such as plasminogen activator, are secreted at high levels by human fibrosarcoma cell lines, but not by non-malignant human fibroblasts. Excreted proteases are widely presumed to have a major role in tumor invasion and metastasis, and proteinase inhibitors have been shown to block tumor formation in vivo (41), and in vitro transformation of rodent cells by carcinogens (42). Therefore, we wanted to see if such proteases were secreted by our MSU-1.1 cell strains that had been transformed into the malignant state by transfection of various oncogenes.

We have carried out a preliminary study of MSU-1.1 cells and their ras-transformed malignant and metastatic variants for the amount of enzyme activity of urokinase plasminogen activator (u-PA) that they secrete, into the serum-free medium and also the amount of enzyme activity that remains bound to the surface of the cells. The results to date indicate that the cells that are malignant have a level of u-PA activity that is significantly (2-fold to 5.4-fold higher than what is found in non-transformed cells). (See Table 1.) There was no correlation with u-PA activity in the medium and the level of enzyme activity is not correlated with the level of expression of the ras oncogenes originally used to transform the MSU-1.1 cells. We plan

Table 1

Urokinase Plasminogen Activator(uPA)

Activity Bound to the Cell Surface or Secreted into the Medium of Cells of the LG1, MSU-1.0, and MSU-1.1 Lineage

Cell Strain	Tumorigenicity	Cell surface bound uPA activity per 10^6 cells	uPA activity secreted into the medium per 10^6 cells
LG1	-	3.6 ± 1.1	58.7 ± 1.5
MSU 1.0	-	5.6 ± 1.0	75.6 ± 17.9
MSU 1.1	-	3.5 ± 0.3	29.8 ± 0.7
E73	-	6.2 ± 0.4	26.3 ± 4.3
L98-2A	-	4.7 ± 0.7	29.9 ± 3.2
3T	+	11.5 ± 1.5	90.9 ± 13.5
5T	+	12.2 ± 0.3	27.2 ± 3.5
8T	+	19.0 ± 3.5	24.2 ± 1.7
2MT1	+	12.4 ± 1.3	46.9 ± 3.9
3MT1	+	9.8 ± 4.2	89.3 ± 8.1
178-2DT	+	20.3 ± 5.1	97.9 ± 28.9
178-2BT	+	20.4 ± 2.8	109.7 ± 21.6
2A-C4MT	+	9.9 ± 1.6	40.2 ± 7.0
L451-BT	+	8.8 ± 1.0	16.4 ± 1.7
L551-3T	+	7.1 ± 1.2	28.8 ± 2.3

to continue these investigations during the renewal period to see if bound u-PA activity can be a marker for malignant transformation of human fibroblasts.

J. Effect of activated ras on the sensitivity of MSU-1.1 cells to ionizing radiation.

In 1988 Sklar (43) reported that several independent NIH3T3 ras transfectants were significantly more resistant to ionizing radiation than the parent 3T3 cells. This result was surprising since ionizing radiation sensitivity is a stable characteristic of cell strains. On rare occasions, radiation-resistant and radiation-sensitive variant mammalian cells have been isolated, but we were unaware of any gene whose expression was correlated with radiation-resistance, such as that reported by Sklar. To see if this phenomenon might apply to human cells, we tested H-ras and N-ras malignantly-transformed MSU-1.1 cell lines for their sensitivity to ionizing radiation compared to that of the MSU-1.1 parental cell line using Co^{60} radiation and the standard ionizing radiation survival curve protocols we previously published (44). If the cells had proven abnormally resistant to radiation, we planned to examine cell strains expressing various levels of mutant ras proteins to see if the degree of radiation resistance correlated with the level of expression of the p21 protein.

The results of our study with MSU-1.1 cells malignantly transformed by transfection of a H-ras oncogene indicated there was no statistically significant difference between the survival curve of parental MSU-1.1 cells and the malignant cell strain derived from it. Therefore, we did not pursue this issue further. In re-examining the results reported by Sklar (43), we concluded that even the difference they observed was not more than a 1.4-fold difference in the slope of the survival curves.

K. Role of growth factor synthesis in malignant transformation by transfected ras genes.

We hypothesized that growth factor synthesis by a cell complements ras expression, then, cells that already synthesize growth factors may be transformable by normal levels of activated (mutated) ras p21 product. As noted in Section A of the series of plasmids carrying an H-ras oncogene tested for transforming activity in our human fibroblast cell strains, only the plasmid engineered for the highest expression of ras proved able to transformed these cells. We proposed to test whether other plasmids in the series (i.e., those with reduced promoters and/or enhancers) would be able to cause malignant transformation of the growth factor independent variant MSU-1.1 cell strains we isolated. For transfection, we utilized a plasmid containing the gpt gene which codes for mycophenolic acid resistance and a T24 H-ras oncogene with an SV40 promoter.

Some cell strains derived from mycophenolic acid resistant colonies exhibited a marked increase in the size and number of colonies that grew in agar. The cells in these populations were somewhat morphologically transformed and expressed the H-ras oncogene at 2 to 3 times normal level. However, as of this time (two months since injection) they have not yet made tumors in the mice.

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VI. PLANS FOR CONTINUING PRESENT OBJECTIVES AND PURSUING THOSE THAT FLOW FROM PAST RESULTS

The work summarized in this Progress Report Section IV Aims A - I has been very successful and opens the way for us to continue with the objectives outlined in the Part One Section II of this Report. The work summarized in this Progress Report under Aims J and K has been completed and will not be continued.

VII. STUDENTS TRAINED AND POSTDOCTORAL TENURES COMPLETED

A. During the past three years, two of the postdoctoral research associates who were supported by this Grant completed their training in the Carcinogenesis Laboratory and accepted permanent positions.

Name of Research Associate	New Position	Date
1. Thomas L. Morgan III, Ph.D.	Staff Scientist Department of Radiological Physics Section Biology and Chemistry Departments Battelle Pacific Northwest Laboratories Richland, WA	8/87
2. Dennis E. Fry, Ph.D.	Staff Scientist Department of Molecular Biology Abbott Laboratories Abbott Park, IL	4/88

B. In addition, three graduate students who were supported by this Grant successfully completed the research for the Ph.D. degree and accepted post-doctoral position elsewhere.

Name of Graduate Student	New Position	Date Relocated
1. Peter J. Hurlin, Ph.D. Department of Biochemistry	Fred Hutchinson Cancer Research Center Seattle, WA	7/88
2. Daniel M. Wilson, Ph.D. Department of Biochemistry	Department of Biochemistry Chemical Industries Institute of Toxicology Research Triangle Park, NC	9/88
3. Robert J. Schilz, Ph.D., D.O. Department of Biochemistry, Medicine	Medical Residency Training Sparrow Hospital Lansing, MI	11/88

C. In addition to these persons who are conducting research supported by this D.O.E. Grant, the following postdoctoral research associates are presently receiving training in the Carcinogenesis Laboratory conducting research supported by other sources of funds. (These persons benefit only indirectly from the past support of this DOE Grant or the predecessor Contract in providing equipment, etc.).

Name of Research Associate		Institution Granting Degree
1.	Nitai P. Bhattacharyya, Ph.D. (Biophysics)	Calcutta University Calcutta, India
2.	Janet Boldt, Ph.D., M.D. (Carcinogenesis) (Medicine)	University of Hamburg Hamburg, West Germany
3.	Geoffrey Curtin, Ph.D. (Cell Biology)	Texas A and M College Station, TX
4.	Marcia Mah, M.D. (Medicine)	Federal University of Minas Gerais Belo Horizonte, Brazil
5.	Dajun Yang, M.D., M.S. (Medicine) (Carcinogenesis)	Sun Yat-sen University Sun Yat-sen University Cancer Institute People's Republic of China
6.	Calvert Louden, D.V.M. (Veterinary Medicine)	Michigan State University East Lansing, MI

VIII. DIVISION OF FEDERAL SUPPORT FOR OVERALL RESEARCH PROGRAMS

The ongoing research program on Carcinogenesis in the Carcinogenesis Laboratory is directed principally by Dr. J. Justin McCormick and is supported by three funding instruments: (1) This **DOE Grant** which is up for competitive renewal; (2) **Contract ES65152** from the National Institute of Environmental Health Sciences; and (3) **Grant CA21289** from the National Cancer Institute. Dr. Veronica M. Maher is co-principal investigator on the DOE Grant and the NIEHS Contract.

NIEHS Contract ES-65152.

Title: "Development of Mammalian Cell Transformation Assay System for Chemical Testing"

Period Funded: 7/1/86-6/30/91*

Total Direct Costs: $197,540 for Year 4

Total Indirect Costs: $82,967 for Year 4

*This contract was originally written as a five year proposal, but must be competitively reviewed each year. It is just beginning Year 4.

NIH/NCI Grant CA21289.

Title: "In Vitro Transformation of Human Cells by Carcinogens"

Period Funded: 7/1/87-6/30/91

Total Direct Costs: $97,808 for Year 3

Total Indirect Costs: $35,639 per Year 3

The NIEHS contract was one of five contracts awarded in July 1986 for the development of quantitative assays for detecting carcinogens by an in vitro mammalian cell transformation assay system. The original purpose was to improve our existing assays and then apply them to the testing of a series of 25 to 40 coded chemicals per year, over a period of five years. The contract supports four persons. In January 1988, when the NIEHS Contract Officers learned of our successful malignant transformation of human fibroblasts and the development of a

series of cell line one step, two steps, three steps away from the malignant state, they altered the purposes of the Contract to encompass treating such cell lines with chemical carcinogens or gene coding for growth factors to determine the mechanisms involved in bringing such cells to malignancy. The revised aims are unique to that Contract and do not replicate work carried out under the NIH/NCI Grant or the DOE Grant. For the information of the reviewers, we have listed the Specific Aims of the NIEHS Contract and the NIH/NCI Grant.

A. Specific Aims of NIEHS Contract ES65152, "Development of Mammalian Cell Transformation Assay System for Chemical Testing".

The research was designed to develop a quantitative assay for the induction of transformation of human fibroblasts to anchorage independence and focus-formation in order to determine whether these assays could distinguish between carcinogens and non-carcinogens in a blind study.

1. The original aims were:

a. PHASE ONE: DEVELOPMENTAL STUDIES:

1) Optimize the conditions for the anchorage independence assay using Chemical A (the 7,8-diol-9,10-epoxide of benzo(a)pyrene) especially to determine if it could be carried out in the absence of serum.

2) Standardize the conditions for exposure of the human cells to various kinds of test chemicals, using four coded chemicals (Chemicals B-E) selected by the Project Officer. These should include two direct-acting compounds and two compounds in need of exogenous metabolic activation. At least one of these compounds should show a pH dependence for activation and/or be volatile.

a. Test each compound for ability to cause cytotoxicity.

b. When conditions for obtaining a cytotoxic dose response have been determined, measure the ability of Chemicals B-E to induce anchorage independence.

3) Using Chemical A and UV radiation, determine whether focus-formation can also serve as an endpoint in quantitative studies of transformation.

4) Using Chemical A and UV radiation, determine whether epidermal growth factor independence (ability to form colonies in serum-free medium lacking EGF) can also serve as an endpoint in quantitative studies of transformation.

5) Investigate the relationships of each of these three transformation endpoints for the ability of the transformants to produce tumors in X-irradiated athymic mice.

b. PHASE TWO: TESTING CODED COMPOUNDS:

1) Test a series of coded chemicals to see if they cause transformation, using anchorage independence as the major assay.

2) Incorporate the other two assays into the test if they prove to be quantitative.

2. <u>The revised aims are:</u>

In January 1988, when the NIEHS Contract Officers learned of our successful transformation of human fibroblasts to the fully malignant state, they requested that we alter our aims to incorporate the newly-developed cell strains as follows:

a. Determine whether malignantly-transformed cells can be produced by chemical carcinogen treatment(s) of the following four non-tumorigenic cell lines:

MSU-1.1; a growth factor independent spontaneous variant of MSU-1.1; MSU-1.1 transfected with the T24 H-ras oncogene and expressing normal levels of the mutant ras protein; and MSU-1.1 transfected with the N-ras oncogene and expressing normal levels of the mutant ras protein.

1) Cells will be treated with the diol epoxide of benzo(a)pyrene (BPDE) or with ethylnitrosourea (ENU), allowed a seven day expression period, and selected for anchorage independent growth, focus formation, growth factor independence, and/or morphological transformation. Cells expressing one or more of the phenotypes will be isolated, grown to large populations and injected into athymic mice to determine whether they are malignant.

2) Malignant tumors will be removed and the cells returned to culture and examined to determine their karyotype, whether the ras genes carry "activating" mutations and whether they synthesize growth factors not synthesized in the non-malignant precursor cells. Part of the tumor will be examined by a pathologist to determine the type of tumor formed.

b. Determine whether malignantly-transformed cells can be produced by transfection of genes coding for the platelet-derived growth factor (PDGF) and the basic fibroblast growth factor (bFGF) into the following three non-tumorigenic cell lines:

MSU-1.1; MSU-1.1 transfected with the H-*ras* oncogene and expressing normal levels of the mutant *ras* protein; and MSU-1.1 transfected with the N-*ras* oncogene and expressing normal levels of the mutant *ras* protein.

1) Cells will be transfected with plasmids containing these genes and selected for anchorage independent growth, focus formation, and growth factor independence. Cells expressing these phenotypes will be isolated, grown to large populations and injected into athymic mice to determine whether they are malignant.

2) Transformed cells will be characterized to show that the transfected gene is present and that the cells make the expected protein product.

b. Malignant tumors will be removed and the cells returned to culture and examined to determine their karyotype, whether the *ras* genes carry the transfected gene and whether they synthesize the appropriate protein growth factor, which is not synthesized in the non-malignant precursor cells. Part of the tumor will be examined by a pathologist to determine the type of tumor formed.

c. Examine RNA from human fibroblasts malignantly transformed by transfection with the H-, K-, or N-*ras* oncogenes using Northern blots to determine whether they synthesize mRNA for growth factors not synthesized in their non-malignant precursor cell line.

d. Determine whether activation of *ras* genes is obligatory for the malignant transformation of fibroblasts, by sequencing the three

ras genes in the two spontaneous malignant transformants we have isolated and in carcinogen transformed cell lines.

B. Specific Aims of NIH Grant CA21289 "In Vitro Transformation of Human Cells"

The overall purpose of this NIH grant was to attempt to induce human fibroblasts to acquire an infinite life span in culture because at the time we wrote the proposal (Fall 1986) the results of our previous research indicated that an infinite life span was a prerequisite if a single cell had to clonally acquire each of a series of changes in a step-wise fashion before it could become malignant. As indicated above in this DOE report, acquisition of an infinite life span seems to represent a major bottleneck in obtaining malignant transformation. Therefore, we decided to concentrate all our effort on generating infinite life span cells into this grant. We proposed:

1. To attempt to induce "immortality" (unlimited life span in culture) in human cells from normal persons, from Bloom's Syndrome patients, and from Fanconi's Anemia patients by exposing such cells to increased oxygen tension.
2. To try to induce "immortality" in such cells by exposing them to the 7,8-diol-9,10-epoxide of benzo(a)pyrene.
3. To examine by reconstruction studies whether non-senescing cells can be eliminated (caused to die) by contact with senescing cells, as a form of "metabolic cooperation".
4. To characterize any existing candidates for "immortalized" human cells to determine whether they exhibit other characteristics of tumor-derived cells, such as growth factor independence or independence from calcium.

5. To characterize fibroblasts derived from fibromas and/or low grade malignancies to determine how they differ from normal cells and from fully malignant cells derived from fibrosarcomas since they may represent intermediates on the path to malignancy.

In the light of our successful generation of the infinite life span MSU-1.0/1.1 cell line, we recently added the following aims to this NIH grant:

6. To attempt to reproduce the creation of an infinite life span human cell line by transfecting the plasmid carrying the myc oncogene into moderately late passage cells and studying them through senescence ("crisis").

7. To determine by cell fusion studies whether the infinite life span characteristic in the MSU-1.0/1.1 cell line is a recessive trait and if so, whether the trait complements that of the various fibrosarcoma-derived cell lines.

[redacted]

NAME: J. Justin McCormick, Ph.D.
TITLE: Associate Dean for Research; Professor, Department of Microbiology, Professor, Department of Biochemistry, and Co-Director, Carcinogenesis Laboratory

[redacted]

EDUCATION

Institution and Location	Degree	Yr. Conferred	Scientific Field
St. Paul's College, Washington, D.C.	B.S.	1957	
St. Paul's College, Washington, D.C.	M.A.	1960	
Catholic University, Washington, D.C.	M.S.	1963	Biology
Catholic University, Washington, D.C.	Ph.D.	1967	Cell Physiology Minor, Radiation Biology

MEMBERSHIP IN PROFESSIONAL SOCIETIES

Sigma Xi
American Association for Cancer Research
American Society for Biochemistry and Molecular Biology
American Society for Cell Biology
Biophysical Society
Tissue Culture Association
Environmental Mutagen Society
American Society for Photobiology
Radiation Research Society
American Association for the Advancement of Science
Society for Risk Analysis

MAJOR RESEARCH INTERESTS

Cellular and Molecular Mechanisms of Carcinogenesis
Chemical and Radiation Carcinogenesis
DNA Repair
Mutagenesis and Transformation of Human Cells
Environmental Toxicology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE

Associate Dean for Research, College of Osteopathic Medicine, Michigan State University, East Lansing, MI, 1987 to present

Professor, Department of Microbiology and Public Health and Department of Biochemistry, Michigan State University, East Lansing, MI, 1980 to present

Professor, Co-Director, Carcinogenesis Laboratory, College of Osteopathic Medicine, Michigan State University, East Lansing, MI, 1980 to present

Associate Professor, Department of Microbiology and Public Health and Department of Biochemistry, Michigan State University, East Lansing, MI, 1978 to 1980

Associate Professor, Co-Director, Carcinogenesis Laboratory, College of Osteopathic Medicine, Michigan State University, East Lansing, MI, 1976 to 1980

Chief, Molecular Biology Laboratory, Division of Biological Sciences, Michigan Cancer Foundation, Detroit, MI, 1973 to 1976

Research Scientist, Michigan Cancer Foundation, Detroit, MI, September 1971 to 1973

Research Associate, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI, 1970 to 1971

Postdoctoral Fellow, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI, 1967-70

Instructor in Biology, St. Paul's College, Washington, D.C., 1963 to 1967

Professor in Biology, St. Peter's Junior College, Baltimore, MD, 1963 to 1966

HONORS

Selected by N.C.I. as a representative of the U.S. at the U.S.-Japan Cooperative Cancer Research Program, Kyoto, Japan, 1977.

Selected by N.I.E.H.S. as a representative of the U.S. at the Fifth US-USSR Symposium on Environmental Concerns, Baku, U.S.S.R., 1978.

Invited by the English Society of Cell Biology and United Kingdom Environmental Mutagenesis Society to present a paper at their joint meeting, 1979.

Member of the Department of Energy's Health and Environmental Research Advisory Committee reporting to the Secretary of the Department, 1989 to present.

PEER-REVIEWED PUBLICATIONS SINCE 1985

M. Watanabe, V. M. Maher, and J. J. McCormick. Excision repair of UV- or benzo[a]pyrene diol epoxide-induced lesions in xeroderma pigmentosum variant cells is "error-free". Mutat. Res. 146, 285-294 (1985).

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[redacted]

NAME: Veronica M. Maher, Ph.D.
TITLE: Associate Dean for Graduate Studies, College of Osteopathic Medicine, Professor, Department of Microbiology and Public Health and Department of Biochemistry and Co-Director, Carcinogenesis Laboratory

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EDUCATION

Institution and Location	Degree	Year Conferred
Marygrove College, Detroit, Michigan	B.S., Biology	1951
University of Michigan, Ann Arbor, Michigan	M.S., Biology	1958
University of Wisconsin, Madison, Wisconsin McArdle Laboratory for Cancer Research	Ph.D., Molecular Biol.	1968
Yale University, School of Medicine New Haven, Connecticut	Radiobiology- Research Associate	1969
Johns Hopkins University, School of Public Health, Baltimore, Maryland	Radiological Sciences- Research Associate	1970

MEMBERSHIP IN PROFESSIONAL SOCIETIES

American Association for Cancer Research
American Society of Microbiology
Environmental Mutagen Society
Tissue Culture Association
Genetics Society of America
American Society for Biochemistry and Molecular Biology
National Association for Women Deans, Administrators, and Counselors

MAJOR RESEARCH INTERESTS

Molecular Mechanisms of Mutagenesis
Role of DNA Repair in Mutagenesis of Human Cells
Molecular Mechanisms of Carcinogenesis
Environmental Toxicology

HONORS

Bacclaurate - Summa cum laude - Marygrove College, Detroit, 1951
NSF Academic Year Institute Scholarship, University of Michigan, Master's Program, 1957-1958
PHS Pre-doctoral Training Grant, University of Wisconsin, Ph.D. Program, 1964-1868
American Men and Women of Science, 1978
Who's Who of America, 1981
Councilor, Environmental Mutagen Society, 1979-1983
Member, Board of Trustees, Marygrove College, Detroit, 1983 to present
Chairperson, Trustees Committee, Marygrove College, 1984 to present
The World Who's Who of Women, 8th edition, 1984-1985
Division of Women's Programs for the Department of Human Relations, Michigan State University
1985 MSU Women's Acheivements Award
Distinguished Faculty Award, 1988

RESEARCH AND/OR PROFESSIONAL EXPERIENCE

Associate Dean for Graduate Studies, College of Osteopathic Medicine, Michigan State University, East Lansing, Michigan, 1987-present

Professor, Department of Microbiology and Public Health and Department of Biochemistry, Michigan State University, East Lansing, Michigan, 1980 to present

Professor, Co-Director, Carcinogenesis Laboratory, College of Osteopathic Medicine, Michigan State University, East Lansing, Michigan, 1980 to present

Associate Professor, Department of Microbiology and Public Health and Department of Biochemistry, Michigan State University, East Lansing, Michigan, 1978 to 1980

Associate Professor, Co-Director, Carcinogenesis Laboratory, College of Osteopathic Medicine, Michigan State University, East Lansing, Michigan, 1976 to 1980

Chief, Carcinogenesis Laboratory, Division of Biological Sciences, Michigan Cancer Foundation, 1973 to 1976

Research Scientist, Department of Biology, Michigan Cancer Foundation, Detroit, Michigan, September 1970 to 1973

Research Associate, Department of Biological Sciences, Johns Hopkins University, Baltimore, Maryland, June 1970 to August 1970

Research Associate, Department of Human Genetics, University of Michigan, Ann Arbor, Michigan, November 1969 to March 1970

Assistant Professor, Department of Biology, Marygrove College, Detroit, Michigan, September 1969 to June 1971

Research Associate, Department of Radiobiology, Yale University Medical School, New Haven, Connecticut, September 1968 to September 1969

Postdoctoral Fellow, Department of Oncology, McArdle Laboratory, University of Wisconsin, Madison, Wisconsin, May 1968 to September 1968

Graduate Student, Department of Oncology, McArdle Laboratory, University of Wisconsin, Madison, Wisconsin, 1964 to 1968

PEER-REVIEWED PUBLICATIONS SINCE 1985

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IX Appendix A

TISSUE PROCUREMENT

E.W. Sparrow, Butterworth, Blodgett, and St. Mary's hospitals are all major affiliate hospitals of Michigan State University. They are acute care facilities, with acitve pediatric services, in Lansing and Grand Rapids. The first three have over five hundred beds each; they are the chief secondary level hospitals in western and central Michigan. A majority of oncology patients are treated at on-site cancer units, or are biopsied before referral to tertiary university centers in Chicago, Ann Arbor, or Detroit. Pathology departments at each hospital have been contacted, and will provide the following tissue samples when available.

1. One square centimeter or more of sarcoma soft tissue will be placed in culture media and refrigerated. These cells, maintained in vitro, will be a primary source of undegraded tumor RNA. An additional portion of tissue will be flash frozen in isopentane and liquid nitrogen for DNA analysis. The sample group will include fibrosarcoma, rhabdomyosarcoma, malignant fibrous histiocytoma, as well as other sarcoma of the extremities, retroperitoneum, head, and neck. We estimate that we will receive two fresh tumor samples per month from the cities of Lansing and Grand Rapids. Since we need only a small number of well-characterized fibroblastic sarcomas to serve as benchmarks in our studies, the number will be sufficient. Histologic slides, including routine H&E and immunocytochemistry preparations, electron micrographs, and all pathology reports will be available to us for verification of tumor type. There will be constant communication between pathologists working in our laboratory and the surgical pathology groups in each hospital; Dr. Jonathan D. Fratkin, who is a visiting scientist in the Carcinogenesis Laboratory of Michigan State University, is a member of the pathology staffs of Sparrow and Butterworth Hospitals. A reliable courier service, which daily visits each of the hospitals and MSU, will be utilized to transport tissues to the Laboratory.

DISEASES OF THE SKIN

TELEPHONE 351-8170

JACQUES HOCHGLAUBE, M.D., P.C.
1090 ABBOTT ROAD
EAST LANSING, MICHIGAN 48823

June 29, 1989

J. Justin McCormick, Ph.D.
Professor, Microbiology and Public Health
and Biochemistry
Co-Director, Carcinogenesis Laboratory
Michigan State University

Dear Dr. McCormick,

I will be happy to continue to provide you with fresh tissue from lesions of Dermatofibroma for your research work. I will be able to do this indefinitely.

Sincerely,

Jacques Hochglaube, M.D.

JH/np

Reprints removed and cycled separately -