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PROGRESS REPORT

Publications Resulting from Research Supported in Whole or in Part by this Grant. (September 1987 through August 1988)

A. Papers in Refereed Journals.

1. 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, 5752-5757 (1987).

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

3. 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, 341-352.

4. P. Hurlin, V. M. Maher, and J. J. McCormick. Malignant transformation of a human fibroblast cell strain, MSU-1.1, by expression of a transfected T24 H-ras oncogene. P.N.A.S. (in press, 1988).

5. 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 (in press, 1988).

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6. 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. (in press, 1988).

7. J. M. Frazier, C. A. Tyson, C. McCarthy, 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. Tox. & Appl. Pharmac. (in press, 1989).

8. D. M. Wilson, D. G. Fry, V. M. Maher, and J. J. McCormick. Stable transformation of normal diploid human fibroblasts by a transfected N-ras oncogene. Carcinogenesis (submitted, 1988).

9. 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-Ki-ras. Oncogene Res. (submitted, 1988).

10. R. J. Schilz, J. E. Dillberger, V. M. Maher, and J. J. McCormick. Growth factor independence of cell lines derived from human fibrosarcomas and human fibroblasts transformed in culture spontaneously or after carcinogen treatment. Cancer Res. (submitted, 1988).

B. Published Abstracts.

1. 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.

2. 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. Cellular Biochemistry Suppl., 12A, 156, 1988.

3. D. G. Fry, L. Milam, J. Dillberger, V. M. Maher, and J. J. McCormick. Malignant transformation by transfection with v-Ki-ras. Amer. Assoc. Cancer Res., 29, 456, 1988.

4. J. J. McCormick, P. J. Hurlin, D. Wilson, J. Dillberger, D. G. Fry, and V. M. Maher. Malignant transformation of human fibroblasts by transfected ras oncogenes. Amer. Assoc. Cancer Res., 29, 455, 1988.

5. 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.

Studies Conducted During Current Year and Results Obtained.

A. Introduction.

There is excellent epidemiological evidence which indicates that carcinogen exposure as a result of lifestyle, work place or even certain medical treatments is causally involved in human cancer. Surprisingly, however, human fibroblasts or epithelial cells cannot be transformed into malignant cells by carcinogen exposure in vitro. The several reports of successful transformation of fibroblasts are the result of contamination of cultures with tumor cells or of using insufficiently stringent criteria for

transformation. This evidence is contained in our 1988 critical review of the field, enclosed as Appendix 1.

Many workers contrast the rarity of successful transformation of human fibroblasts to malignancy with the ease at which the process occurs in rodent fibroblasts of some species. There is evidence that rodent fibroblasts can be malignantly transformed by carcinogen exposure in culture. There are, however, no quantitative methods to malignantly transform a normal rodent cell with one or more treatments with carcinogen. Rather, the vast majority of studies make use of rodent fibroblast cell lines (e.g., 10T-1/2, 3T3, etc.) that have an infinite lifespan and perhaps other transformed phenotypes, and after carcinogen treatment a small subpopulation of these cells are transformed to express a new phenotype, such as focus formation, which correlates with tumorigenicity.

Human fibroblasts exhibit many different transformed phenotypes (anchorage independence, focus formation, etc.) as a result of carcinogen treatment. A recent special issue of **Mutation Research** on human fibroblast transformation that we have edited presents extensive evidence for this. However, the cells are <u>not</u> malignantly transformed when they express any of these phenotypes. There is one transformed phenotype, infinite lifespan, that differs from the others in that it has only very rarely been induced by carcinogen treatment. Namba has reported that two times in approximately 250 experiments, infinite lifespan cells arose. This phenotype has never been observed to occur spontaneously despite the fact that there are thousands of papers that have utilized human fibroblasts. After SV40 transfection or infection, an infinite lifespan cell strain is sometimes observed to arise. However, this is also a rare event since all human fibroblast cultures are readily transformed, but only rarely ($\sim$20 times in the world literature) have infinite lifespan

variants been reported to arise in such cultures. SV40-transformed cells are not malignant and do not spontaneously give rise to malignant variants. Furthermore, SV40 is a monkey virus and there is no evidence that it has a role in human carcinogenesis. Cells from human malignant tumors frequently give rise to infinite lifespan cell strains. This is not to say that all tumors give rise to infinite lifespan cell lines. Many, in fact, die out after a period in culture. This may be caused by inadequate nutrition, etc. However, the most important observation is that many human tumors give rise to infinite lifespan cell strains, an observation never made when cells from normal human tissues are put in culture.

The most obvious role for the infinite lifespan phenotype in malignant cell transformation is usually overlooked. If one assumes that to become malignantly transformed, a human fibroblast in culture must acquire by clonal selection two or more new phenotypes, then the extended or infinite lifespan phenotype is needed to allow the cell to gain the new phenotypes and to form a tumor large enough to be life-threatening. The empirical basis for this postulate is found on page 286 of paper #1 in Appendix 1. reprint removed, ds

We have developed an infinite lifespan human fibroblast cell line after transfection of a v-myc gene into human fibroblasts. The cells have normal morphology, have doubled more than 200 times since going through crisis, are not tumorigenic, and except for their infinite lifespan, are apparently normal. These cells can be converted into malignant cells by transfection and expression of a H-, N- or K-ras oncogene. When finite lifespan human fibroblasts are transfected with the same ras gene constructions, the cells take on the same in vitro characteristics, but do not form tumors and they still retain the finite lifespan characteristic. Since the ras and myc oncogenes used to transfect the MSU-1.1 cells have normal proto-oncogene

homologs in the human genome, it seems likely that it is these normal genes that become "activated" by carcinogen attack. We have undertaken the transfection studies to understand the role of various oncogenes in malignant transformation of human fibroblasts. With a knowledge of the phenotype caused by a specific oncogene, we can replace the transfection of a specific oncogene with carcinogen activation of the homologous gene. By proceeding in this way, we should be able to develop qualitative methods for the malignant transformation of human fibroblasts by carcinogens.

B. Transformation of Fibroblasts by Transfected ras Oncogenes.

The essential data on the malignant transformation of the MSU-1.1 human fibroblast cell strain by transfection of the H-ras oncogene is described below in the Abstract from a paper now in press in **PNAS** (Appendix #2). preprint removed.

"We showed previously that normal diploid human fibroblasts that express a transfected T24 H-ras oncogene exhibit several characteristics of transformed cells, i.e., altered morphology, anchorage independence, and focus-formation, but do not acquire an infinite lifespan and are not tumorigenic. To extend these sudies of the transforming ability of the T24 H-ras oncogene in human cells, we have utilized an infinite lifespan, but otherwise phenotypically normal, human fibroblast cell strain, MSU-1.1, developed in this laboratory following transfection of diploid fibroblasts with a plasmid carrying a v-myc oncogene. This cell strain has a stable, near-diploid karyotype. Transfection of MSU-1.1 cells with the T24 H-ras oncogene flanked by 2 transcriptional enhancer elements (pHO6T1) yielded foci of morphologically transformed cells. No such transformation occurred if the plasmid containing the T24 H-ras oncogene had only one enhancer or none at all, or if the non-mutant cellular H-ras gene was transfected in the pHO6 vector. Cell strains derived from such foci expressed high levels of T24 H-ras p21, formed colonies in soft agar at high frequency, proliferated rapidly in serum-free medium containing 0.1 mM calcium, which does not support growth of the parental cell line, and formed progressively-growing, invasive fibrosarcomas in athymic mice. These foci-derived T24 H-ras-transformed cell strains had the same karyotype as the parental MSU-1.1 cells and the cells from the tumors derived from them also exhibited a karyotype identical to that of the parental MSU-2.2 cells. Transfection of the T24 H-ras oncogene in pHO6T1 into two

other infinite lifespan human fibroblast cell lines, cells that had not been transfected with v-myc, also resulted in malignant transformation, suggesting that the infinite lifespan phenotype of MSU-1.1 cells, and not necessarily expression of the v-myc oncogene, was the factor that complemented T24 H-ras expression to cause malignant transformation."

In the above study, Hurlin et al., showed that the H-ras-transformed MSU-1.1 cell strains, as well as the other two H-ras transformed infinite lifespan cell strains overexpress the transfected ras gene. (See Fig. 4 of the manuscript by Hurlin et al. in Appendix 2.)

In this study, the H-ras transfected MSU-1.1 cells were selected by focus formation. Cells transfected with the same mutant ras gene, in a vector that contained one enhancer instead of two, did not form foci. This suggests that overexpression of the mutant ras protein is required for focus formation and perhaps, for tumorigenicity. Parallel experiments with the MSU-1.1 cells were carried out with a plasmid carrying the N-ras gene. The N-ras-transfected MSU-1.1 cells formed foci, were malignantly transformed, and overexpressed the N-ras protein. When MSU-1.1 cells were transfected with a plasmid carrying the viral K-ras gene, these also formed foci and were malignantly transformed. However, the cells expressed the viral ras gene at normal levels. These results were somewhat surprising. The explanation may be that the viral K-ras oncogene is known to have mutations at codon 12 and at codon 60. Activated H-, N- and K-ras genes isolated from tumors ordinarily have a mutation only at codon 12 or codon 60. The enhanced transforming ability of the viral K-ras gene may be the result of its having the two mutations. It should be noted that the viral K-ras gene always exhibits some minor differences in sequence when compared with the cellular K-ras gene of the rat, the species of origin. Whether these differences have any significance is not known.

Since the objective of these experiments on ras transformation was to determine how the endogenous proto-oncogenes are modified in tumorigenenesis, we examined ras expression in six human fibrosarcoma-derived cell lines to determine whether these cells also overexpress the ras protein. Immunoprecipitation data on the p21 ras protein are shown in Fig. 1. It is readily apparent that none of these cells express as a high level of ras protein as did the H-ras, or N-ras-transfected MSU-1.1 cells. This indicates that, although overexpression of ras protein could play a role in malignant transformation of human fibroblasts, it is not the common mechanism for such transformation. This study shows the need of verifying the phenotypic changes achieved by transfection by comparing them to human tumor-derived cells.

In fact, these results should not be surprising since point mutations in codons 12 or 60, and not overexpression, have been shown to be the common mechanism of ras activation. We now know from the work of Brown, et al., (1984) and the recent study of Andeol, et al., 1988 that two of these six fibrosarcoma cell lines have such mutation-activated ras genes. A mutation in the HT1080 cellular N-ras gene caused a marked shift in the electrophoretic mobility of the N-ras protein (see 3rd lane from the right in Fig. 2 where a distinct band running below the two ras p21 bands can be observed. The other ras mutation causes no shift in electrophoretic ability, which clearly indicates that other methods must be used to detect ras mutations (Andeol, et al., 1988). Fortunately, there now are new methods to determine with a high level of certainty whether ras genes are activated.

An important question raised by our ras oncogene transfection studies is whether MSU-1.1 cells represent a unique human fibroblast cell strain which is more readily transformed by transfected ras genes than other cells would be. We, therefore, determined whether two other infinite lifespan, but

non-tumorigenic, human fibroblast cell linescould be malignantly transformed with the same pHO6T1 ras-containing plasmids. For this purpose we chose the KMST-6 cell line of Namba and the SV40 transformed cell line GM637. Following transfection, both of these cell lines were selected for drug resistance.Cell populations were isolated and these were injected into athymic mice. Malignant fibrosarcomas resulted. The implication of this study is that it is the infinite lifespan phenotype complements the expression of a mutant ras protein to bring about malignant transformation. There may, of course, be additional changes required for malignancy that were acquired spontaneously.

C. Analysis of Human Fibrosarcoma-Derived Cell Lines for the synthesis of Growth Factors.

Any theory of carcinogenesis must explain why malignant cells replicate whilst adjacent normal cells do not. Sporn and Todaro suggested that if tumor cells synthesized their own growth factors, such proteins could drive a cell's replication. An enormous literature has developed showing that many different types of tumor-derived and other transformed cells do in fact synthesize their own growth factors. The literature on cell transformation suggests that growth in vitro independent of exogenous protein growth factor is the phenotypic expression of this property.

To test this hypothesis, we examined six human fibrosarcoma cell lines to determine whether they would replicate in vitro under conditions in which normal human fibroblasts would not replicate, i.e., in medium with reduced calcium without exogenous protein growth factors (Fig. 2). The fibrosarcoma cells could be divided into two groups, I and II. Cells in group II were not stimulated to replicate by any exogenous growth factor. Since these cell lines were already doubling once every 24 hours, the most likely explanation is that they already were doubling at the maximal rate. Cells in group I replicated slowly without exogenous growth factors, but responded to growth factors by increasing their rate of replication.

Most interestingly, the rate of tumor growth in athymic mice parallels the amount of growth factor synthesized, i.e., the cells of group II make a 1 cm tumor in approximately 15 days, whereas those of group I made a tumor in 30-40 days. One exception to this finding is that SW982 cells have never been observed to form a tumor.

The simplest explanation for these results is that the fibrosarcoma-derived cells synthesize their own protein growth factors, whereas normal fibroblasts do not. We decided to address this problem by determining if the mRNA fraction of the fibrosarcoma cells contained the message for growth factors which were absent in the normal fibroblast. This experiment is not quite complete, but a coherent picture is already emerging (see Table 1). The six growth factors at the top of the chart are each able to drive replication of normal human fibroblasts when added exogenously. IGFII and TGF-α were studied for the sake of completeness but have no effect on replication when added exogenously. The fibrosarcoma-derived cells of group II clearly synthesize higher levels and more growth factors than the cells of group I. The normal fibroblasts synthesize only low levels of (A)PDGF. This data is consistent with the growth of the cells in serum-free medium reported above. The one exception is that the tumor cell line known as NCI synthesizes the same type and level of growth factor as control fibroblasts. Perhaps the NCI cells synthesize a known growth factor, such as thrombin, that we do not have a probe, for or perhaps some growth factor not yet discovered. They may also have some other aberration in a growth factor pathway.

These studies indicate clearly that endogenous synthesis of mRNA for growth factors and presumably the protein is likely to play a causal role in the carcinogenesis process in fibroblasts. Parallel studies are underway with our various MSU-1.1 derivative cell strains that are tumorigenic.

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TABLE 1

Expression of Growth Factor mRNA in Human Fibroblasts and Human Fibrosarcoma-Derived Cells as Determined by Northern Blot Analysis

	Normal Fibroblasts				Group I Fibrosarcoma Cells			Group II Fibrosarcoma Cells				
	NF812	SL68	KD	SL68 serum	SW 982	NCI	SW 684	SHAC	HuT-14	HuT-12	HT1080	VIP:FT
(A)PDGF	1	1	1	1	1	1	2	4	4	4	1	1
(B)PDGF	0	0	0	0	±	0	0	1	1	1	1	1
TGFα	0	0	0	0	0	0	0	2	2	2	2	1
EGF	0	0	0	0	0	0	0	0	0	0	0	0
aFGF	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
bFGF	0	0	0	0	0	0	0	4	0	0	0	0
IGF-II	0	0	0	0	0	0	0	1	0	0	0	0
TGF-β	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Abbreviations:

(A)PDGF	A chain of Platelet-Derived Growth Factor.
(B)PDGF	B chain of Platelet-Derived Growth Factor.
TGFα	Transforming Growth Factor alpha.
EGF	Epidermal Growth Factor.
aFGF	Acidic Fibroblast Growth Factor.
bFGF	Basic Fibroblast Growth Factor.
IGF-II	Insulin-like Growth Factor II.
TGF-β	Transforming Growth Factor beta.
N.D.	Not Done.

Number in table indicates the relative amount detected, 1 is just detectable, 4 is the highest amount detected.

FIGURE LEGENDS

Figure 1. Expression of the ras p21 protein in various cell lines. The ras encoded p21 proteins were immunoprecipitated with antibody from [^{35}S]methionine-labeled cell lysates and analyzed by electrophoresis in 12.5% SDS-NaDodSO$_4$ polyacrylamide gels and flourography. The location of the endogenous ras encoded p21 protein doublet is indicated by the bars. Starting at the left, the first column is from normal human fibroblasts, the second from MSU-1.1. Columns 3-10 are from human fibrosarcoma-derived cell lines or other sarcoma-derived cells. Column 11 is the human bladder carcinoma cell line T24 from with the T24 ras gene was isolated, column 12 are normal human fibroblasts. Only the NCI and T24 cell lines appear to overexpress p21 ras proteins and these have only a little above normal levels.

Figure 2. Effect of growth factors, serum and calcium on the replication of fibrosarcoma-derived cell lines of group I (Panels C and D), fibrosarcoma-derived cell lines of group II (Panels E and F). Cells were plated into culture dishes at a density of 0.7 - 2.2 x 10^3 cell/cm^2 12-16 h prior to determining the initial cell numbers on Day 0. Plating medium was then replaced by McM medium containing 1 mM calcium supplemented with 10% fetal bovine serum (○) or growth factor assay medium containing no growth factors (▲), 1.5 ng/ml PDGF (△), 3 ng/ml EGF (●), 10 ug/ml insulin (□), or 1 mM calcium (■). The media were replaced with freshly prepared test media after 3-4 d as indicated by arrows (↑). Each point represents the average of duplicate dishes.

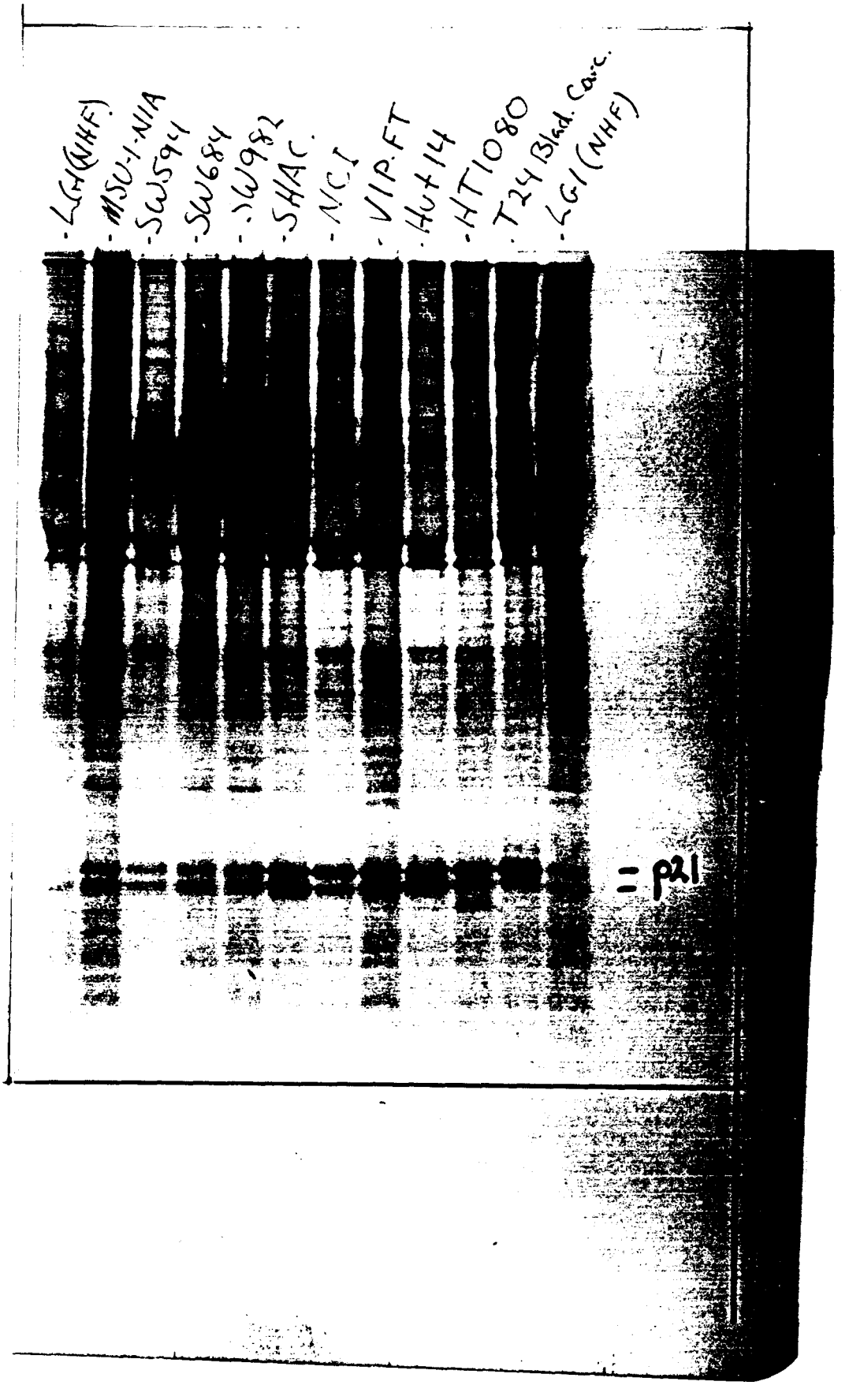
-LG1(NHF)
-MSU-1-NIA
-SW5941
-SW684
-SW982
-SHAC.
-NCI
-VIP-FT
-Hut14
-HT1080
-T24 Bld. Ca.c.
-LG1(NHF)
= p21

FIGURE 1

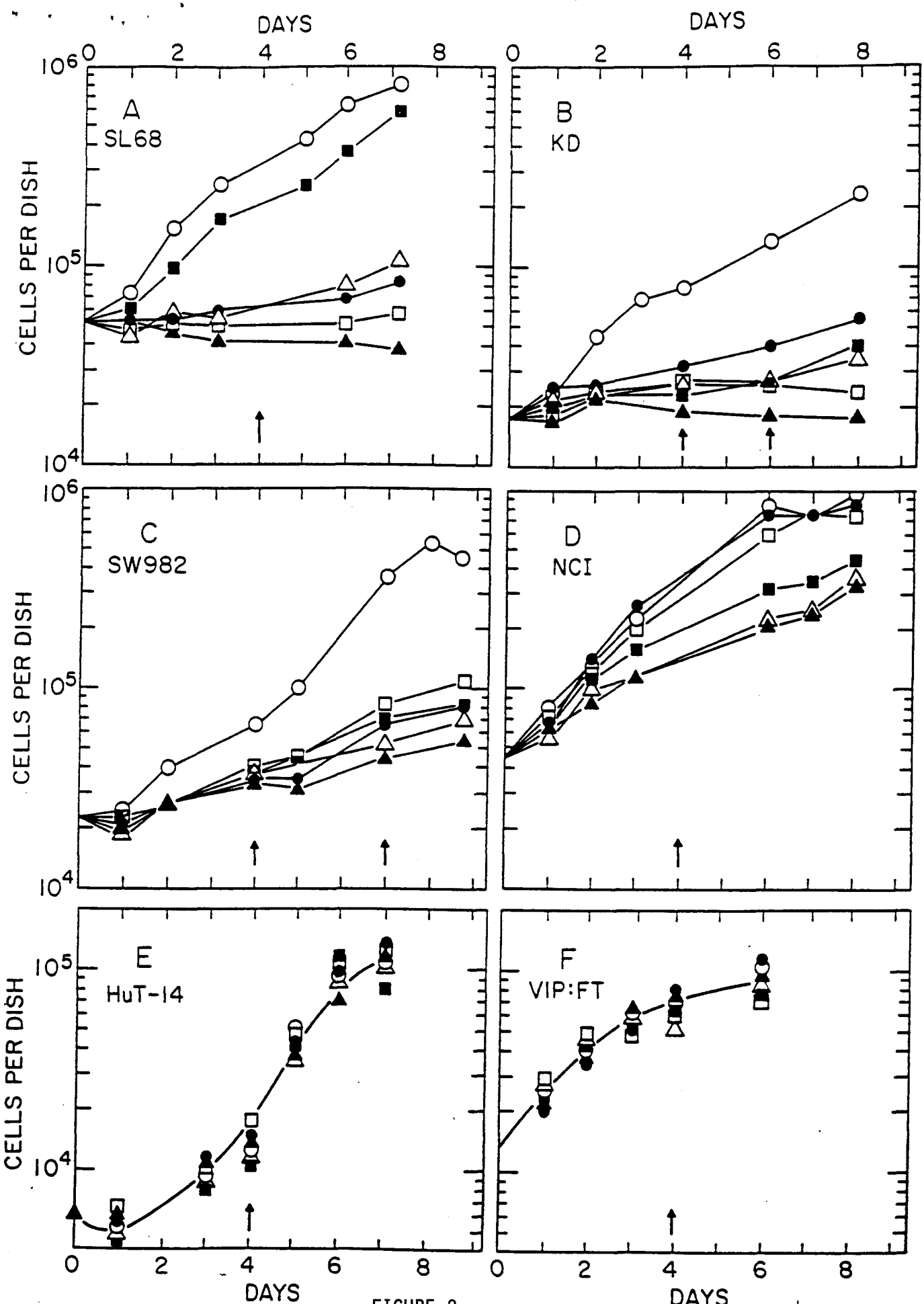

FIGURE 2

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