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MUTATION CARRIERS : LEADERED BY SPANIARDS US LABORATORIES IN THE EARLY 80^s

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ABSTRACT.-

The history of the initial protocols to transfer viral genetic products inducing tumors was greatly facilitated by the availability of continuous cell line of highly contact-inhibited cells (NIH/3T3). During the 70^s the teachings of those involved in this research reached some of the recent arrived in the US Spanish biologists. And in the early 80^s a significant group of Spanish oncologists developed laborious techniques for the detection of oncogenes, involving the gene cloning of regions where are aberrations, sequencing and identification of structural genes in the affected loci and then determination of their role in cancer. Pellicer worked 1976-80 in cell proliferation & cancer at the Columbia University's Medical Center, College of Physicians and Surgeons. In 1979, Manuel Perucho went to a Gordon Conference on Immunology in New York. Ángel Pellicer communicated him, his transfection results. It was the why Perucho moved away from Berlin Max Planck Institut für Molekulare Genetik to US CSHL and later to Stony Brook. Also in 1979, Mariano Barbacid, from the Laboratory of RNA Tumor Viruses, NCI, Bethesda went to New York, where Ángel Pellicer communicated him his transfection results. These three molecular oncology research leaders met with oncogene. After the initial observation that a significant proportion of the tumors scoring positive in the fibroblasts used – the NIH 3T3 cells – and that the genes responsible belonged to the ras family, Ángel Pellicer isolated the two main murine genes responsible for the phenotype, N-ras, in results presented in 1984. The following year he sequenced the complete coding region of N-ras, and suggested evidence for the spectrum of activated mutations in different mouse strains and by different agents. A number of methodical factors affect this historical series of performances. Concept symbols derived from the display of the graphical distribution along time of the concurrent performances, reveal the successive shifts of their authors from the Columbia University to the Cold Spring Harbor Lab and the Frederick Cancer Research Center.

INTRODUCTION.-

In a first approach, the integrated interaction of the viral and cellular genomes is due to L.A. Zilber (1894-1966),¹ the theory of oncogenes was originated by R. Huebner (1914-1998) and G. Todaro (1937-),² H. Temin (1934-1994)³ elaborated the theory of the provirus, D. Baltimore (1938-) isolated the enzyme transcriptase inverse, and M. Barbacid (1949-) activated the first oncogene.⁴ The most accurate reconstruction from the true ancestral cancer immunologist Zilber, may take into account of the importance of the use of cell lines free of sarcoma virus in mutagenic experiments.

In 1969, was segregated the third line 3T3, from the mice embryos in the Swiss/NIH line. This line was to be called NIH 3T3 (after the name of the National Institute of Health where they proliferated). One of the authors was Todaro.⁵ The cells gave 20-25 steps to be cloned, until achieving the clone (clone 6) with a particularly high plating efficiency and low saturation density.⁶ Later the NIH 3T3 was used mainly to study the viral transformation in tissue- and cell- cultures. The selection of this line for transfection experiments is conditioned by a high efficiency in the integration and fixation of the exogenous DNA in the genome of the NIH 3T3 cells.

The considerable interest that had the discovery that an ample variety of tumor cells contained activated *ras* genes, was detectable through gene transfer in NIH 3T3 cells, and displays the work by Barbacid in August 1983.⁷

This work was presented to the US Natl Acad Sci on April 1983 by the 1953 Nobel Prize, Severo Ochoa, who also selected to present there on July 1985 a development on human adenocarcinoma cells of previous NIH 3T3 results, associated to the detection of point mutations authored by M. Perucho.^{8,9} The point mutation malignant acquisition responsibility discovery was the why after which Barbacid was awarded in 1988,¹⁰ but a carcinogenic induction of *ras* oncogene activation was also obtained under the responsibility of A. Pellicer, being I. Guerrero his postdoc.¹¹

¹ Silber, L. (1923) "Ueber das Wesen der Weil-Felixschen Reaktion". Centralbl. f. Bakt. etc. 1. Abt. Originale. Bd. 89. Heft 7/8. pp.250-259, where is proved the serological modification experienced by the bacteria *Proteus vulgaris*, hereditary transformed into a variant that is agglutinated by the serum of the guinea pig infected by louse-borne typhus virus. A precedent of the experiments by Griffith on the transformation of pneumococci by way of transfer from a virulent culture. See Griffith, F. (1928) "The Significance of Pneumococcal Types". Journal of Hygiene, Vol. 27, Iss. 2, p. 113.

² Huebner RJ, Todaro GJ. (1969) "Oncogenes of RNA tumor viruses as determinants of cancer". Proc Natl Acad Sci U S A., Vol. 64, N° 3, pp. 1087-1094.

³ Herzberg, M.; Revel, M. (1973) Atlas de biología molecular. Barcelona, Omega.

⁴ Garte, S.J.; Hochwalt, A.E. (1989) "Oncogene activation of experimental carcinogenesis: the role of carcinogen and tissue specificity". Environmental Health Perspectives, Vol. 81, pp. 29-31.

⁵ Todaro and Green were the precursors of 3T3 cells. Todaro, G.J.; Green, H. (1963) "Quantitative studies of growth of mouse embryo cells in culture and their development into established lines". Journal of cell biology, Vol. 17, Iss. 2, pp. 299-313.

⁶ Jainchill, J.L.; Aaronson, S.A.; Todaro, G.J. (1969) "Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells". Journal of Virology, Vol. 4, Iss. 5, pp. 549-553. (p. 550.)

⁷ Santos, E.; Reddy, E.P.; Pulciani, S.; Feldmann, R.J.; Barbacid, M. (1983) "Spontaneous activation of a human proto-oncogene". Proc Natl Acad Sci USA, Vol. 80, N° 15, pp. 4679-4683.

⁸ Winter, E.; Yamamoto, F.; Almoguera, C.; Perucho, M. (1985) "A method to detect and characterize point mutations in transcribed genes: Amplification and overexpression of the mutant c-Ki-*ras* allele in human tumor cells". Proc Natl Acad Sci USA, Vol. 82, N° 22, pp. 7575-7579.

⁹ In 1981, Perucho provided a powerful technique for efficiently transform NIH 3T3 cells, see Perucho, M.; Goldfarb, M.; Shimizu, K.; Lama, C.; Fogh, J.; Wigler, M. (1981) "Human-tumor-derived cell lines contain common and different transforming genes". Cell, Vol. 27, Iss. 3 Pt 2, 467-476.

¹⁰ The "cancer Nobel" was attributed (in 1988) by the Switzerland Josef Steiner Foundation to Barbacid "for providing evidence that the interaction of a carcinogen with a *ras*-proto-oncogene creates the event that initiates tumor formation". Reddy, E.P.; Reynolds, R.K.; Santos, E.; Barbacid, M. (1982) "A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma". Nature, Vol. 300, pp. 149-152. For a meticulous figure

Ángel Pellicer, professor in the NYU Dpt of Pathology from 1980, worked with animal model systems of carcinogenesis, by using *ras* genes because of his great clinical relevance. Constantly in contact with Spain through the training of postdocs, he admitted Isabel Guerrero as a Fellow of the Spanish High Research Council in 1983.¹² The discovery was that a significant proportion of the tumors scored positive in the NIH 3T3 focus forming assay, and the genes responsible belonged to the *ras* family. After the initial observation, the two main murine genes responsible for the phenotype, *N-ras* and *K-ras* were quickly identified. The following year Pellicer sequenced the complete coding region of *N-ras*, identified the activating mutation and indicated that in some tumors there was loss of the normal allele, suggesting that the wild type copy (proto-oncogene) was exerting some restraining effect on the action of the activated version (oncogene). Further studies continued providing evidence for the spectrum of activating mutations in different mouse strains and by different carcinogenic agents.

The Spanish team with which M. Barbacid solved the question that served to establish the molecular basis of human neoplasia was formed by E. Santos, D. Martín Zanca, and V. Notario. The Spanish research group conducted by A. Pellicer involved V. Corces, P. Calzada, A. Villasante, and A. Pellicer. M. Perucho by working with a team that included as Spanish collaborators C. Almoguera, C. Lama and J. Jordano afforded with the characterization of human and murine cellular oncogenes and provided a method to detect mutations in transcribed genes. A previous communication to this conference reported on the constitutional history of these three groups.¹³

But all these experiments arrived to function just because of the fortunate circumstance, that the NIH3T3 cells were not normal at all, but that they already have accumulated a great number of genetic alterations subjacent to the gradual process of neoplastic transformation. They need one last push, provided by the dominant oncogene.

SCIENTIFIC AGENDA OF ÁNGEL PELLICER.-

The Spanish immunological tradition, on the base of technical applications of these cells, started with J. Salas¹⁴ the doctoral advisor of M. Perucho, on DNA binding proteins from hamster fibroblasts, normal or transformed by oncogenic retroviruses. Salas was involved during his post-doctoral fellowship at NYU, in the early seventies, with H Green, the creator with GJ Todaro of this cell line.¹⁵

depicting the importance of this last paper see Morange M. (1997). "From the regulatory vision of cancer to the oncogene paradigm, 1975–1985". *J. Hist. Biol.*, Vol. 30, pp. 1–29. (Fig. 1, p. 21).

¹¹ Guerrero, I.; Villasante, A.; Corces, V.; Pellicer, A. (1984) "Activation of a c-K-*ras* oncogene by somatic mutation in mouse lymphomas induced by gamma radiation". *Science*, Vol. 225, pp. 1159-1162.

¹² Other postdoctorals with prof. Pellicer have been Claudette Boni, Alfredo Villasante, Javier Leon, Elizabeth Newcomb, Joan Berman, Timothy Thomson, Montserrat Corominas, Ramon Mangues, Renata Schiavo, Guillermo Saez, Rosario Oliva, Javier Santos, Antonio Garcia-Espana, Juan Sanchez-Arias, Marcos Malumbres, Neus Ferrer, Su-Ying Lu, Quixia Lan, Inmaculada Hernandez, Yixing Li, Ignacio Perez de Castro, Laura Martello-Rooney, Marta Benet, and Raffi Suzme.

¹³ Currás, E.; Wulff Barreiro, E. (2008) "Integration in Europe of human genetics results obtained by Spaniards in the USA: a historical perspective". *Scientometrics*, Vol. 75, Nr. 3, pp. 473-493.

¹⁴ Pollack, R.; Salas, J.; Wang, R.; Kusano, T.; Green H. (1971) "Human-mouse hybrid cell lines and susceptibility to species-specific viruses". *Journal of Cellular Physiology*, Vol. 77, Iss. 1, pp. 117-119. Salas, J.; Green, H. (1971) "Proteins binding to DNA and their relation to growth in cultured mammalian cells". *Nature: New biology*, Vol. 229, pp. 165-169.

¹⁵ On the laboratory of Howard Green as where George Todaro developed the 3T3 lines, see Teebor, G.W. (s.a.) "History of the Department of Pathology of the New York University School of Medicine".

In the middle eighties, Ángel Pellicer research agenda is represented in Fig. 1.¹⁶ The general topology of this picture differentiates the upper zone with the methods used along the researches, from the down zone with aims and results obtained. As observed by the use of colours, all the articles were the result of research supported by governmental programs. Criteria of gene purification and cell-transformation observation on animals, like mice, served to experiment with mutations in oncogenes. Less highest intensity visualization of terms, as the corpus is associated with dates reflects superimposed information that reflects less disseminated researches. The structure of the tree is hidden in the center but the clades obtained at the two tips of the main branch substantially shows the chemics vs. radiation carcinogenic procedures.

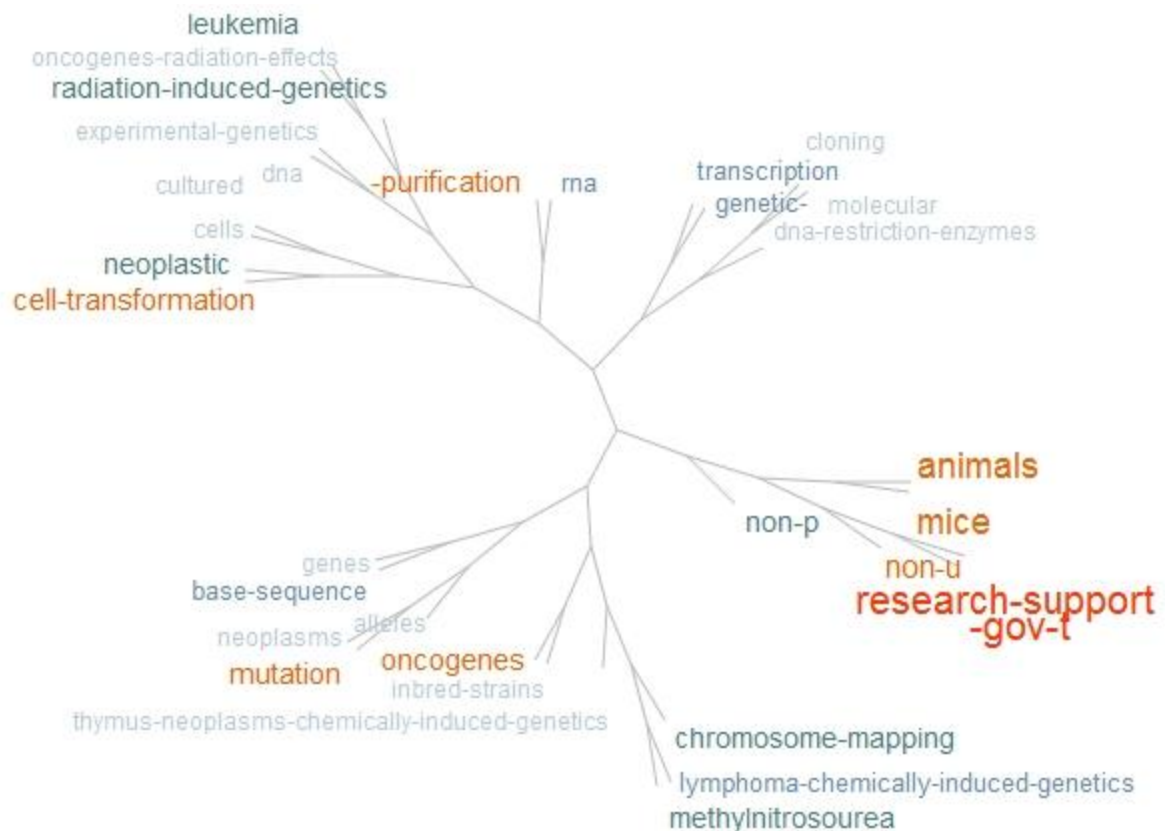


Fig. 1. Maximum likelihood unrooted tree of the tumors scored positive and the genes responsible of it, discovered by Ángel Pellicer.

With Ángel Pellicer¹⁷ it must be said that “until 1977, the methods of gene transfer were successful only when DNA of low complexity was used”, and that “subsequent improvements made the transfer feasible using total vertebrate and mammalian”. Pellicer’s historical three viral positive thymidine kinase colonies were obtained by using the mouse Ltk- cell line, one of the cell lines at this date that possessed exogene DNA incorporation and subsequent stabilization efficiency enough high to permit the gene transfer by using total cellular DNA, with the efficiencies reached by Michel Wigler modifications of the original method. The other line was NIH 3T3. The experiment by Pellicer

¹⁶ A maximum likelihood (ML) analysis was carried out with Treecloud, applying semantic proximity algorithms, see Gambette, P.; Véronis, J. (2009) "Visualising a text with a Tree Cloud". IFCS'09: International Federation of Classification Societies Conference.

¹⁷ Pellicer, A. (1986) “Gene purification by transfection methods”. In: Kucherlapaty, R., ed. Gene transfer. Plenum Press, p. 264 (ref. in vid. nt. 37).

conclusively attained the theoretical efficiencies of the minimum value in need to transfer a single copy gen; and in contrast with the first successes of the sixties this experiment was reproducible.¹⁸

A result gene transfer by using total mammalian DNA was obtained by Pellicer in the Columbia University in 1978.¹⁹ Predoc relationships in Madrid were crucially extended by Pellicer at Columbia on transfection protocols. As in 1979, Pellicer received Barbacid from Bethesda and Perucho from Europe in New York, and he transcribed for both his friends the "ownership" of the transfection experiments in the NIH 3T3 cell lines. The next year (1980) came with Pellicer's as tenured professor at the NYU Dpt. of Pathology, a tenure in the NYU at Stony Brooks for Perucho and a new lab. of cellular and molecular biology at NCI with Barbacid at its head.

Once they knew what they wanted from Pellicer, in 1979, how to make growth fibroblasts after successive transfer associated with malignant properties in vivo by using NIH 3T3 cells ... all three molecular oncology research groups leadered by Spaniards decided to identify and isolate human oncogenes through the use of transfection techniques.

In 1982, both the groups of Mariano Barbacid and Eugenio Santos within the National Cancer Institute and of Michael Wigler and Manuel Perucho at the Cold Spring Harbor Laboratory found the oncogene of the T24 line of human bladder carcinoma cells. For his part, Pellicer decided to work in the identification and isolation of murine oncogenes also using transfection techniques in his own labo at NYU Dept of Pathology from 1980.

In the heat of the argument developed by Szybalski²⁰ and through his involvement in the integration by cotransformation of all the donor cell genome into recipient chromosomes, the question of whether or not spontaneous neoplastic transformation of 3T3 cells were responsible for unstability by transfection led to met oncogene.

Created in the context of 3T3 cells and at the same time, as a reference scenario for how experiments can "go",²¹ 3T6 cells (a line established by transfer of 3×10^5 cells to new capsules each six days), were used in 1980,²² in an ongoing study of molecular genetics conducted since 1978²³ at the Molecular Biology Center, Autonomous University in Madrid. The Jesús Ávila group worked in the characterization and analysis of the neuronal proteins associated to a component of the cytoskeleton,

¹⁸ Wigler, M; Pellicer, A; Silverstein, S; Axel, R. (1978, Jul.) "Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor". Cell, Vol. 14, Nr. 3, pp. 725-731.

¹⁹ Perucho, M. (1994) "La transformación genética de eucariotas superiores y su influencia en el descubrimiento de los oncogenes humanos". In: Casadesús, J.; Ruiz Berraquero, F. (eds.) (1994) Descifrar la vida. Sevilla, Universidad. pp. 261-282.

²⁰ In 1964, Szybalski reported, by working with a single cell derivative of the Detroit-98 line, originally obtained by nonmalignant human sternal puncture, that the mutant phenotype, the obtention of malignant clones, is determined by the tissular differentiation specific conditions. See Szybalski, W. (1964) "Chemical reactivity of chromosomal DNA as related to mutagenicity: studies with human cell lines". Cold Spring Harb Symp Quant Biol., Vol. 29, pp. 151-159.

²¹ Knorr-Cetina, K.; A mann, K. (1990) "Image dissection in natural scientific inquiry". Science, Technology and Human Values, Vol 15, Iss. 3, pp. 259-283.

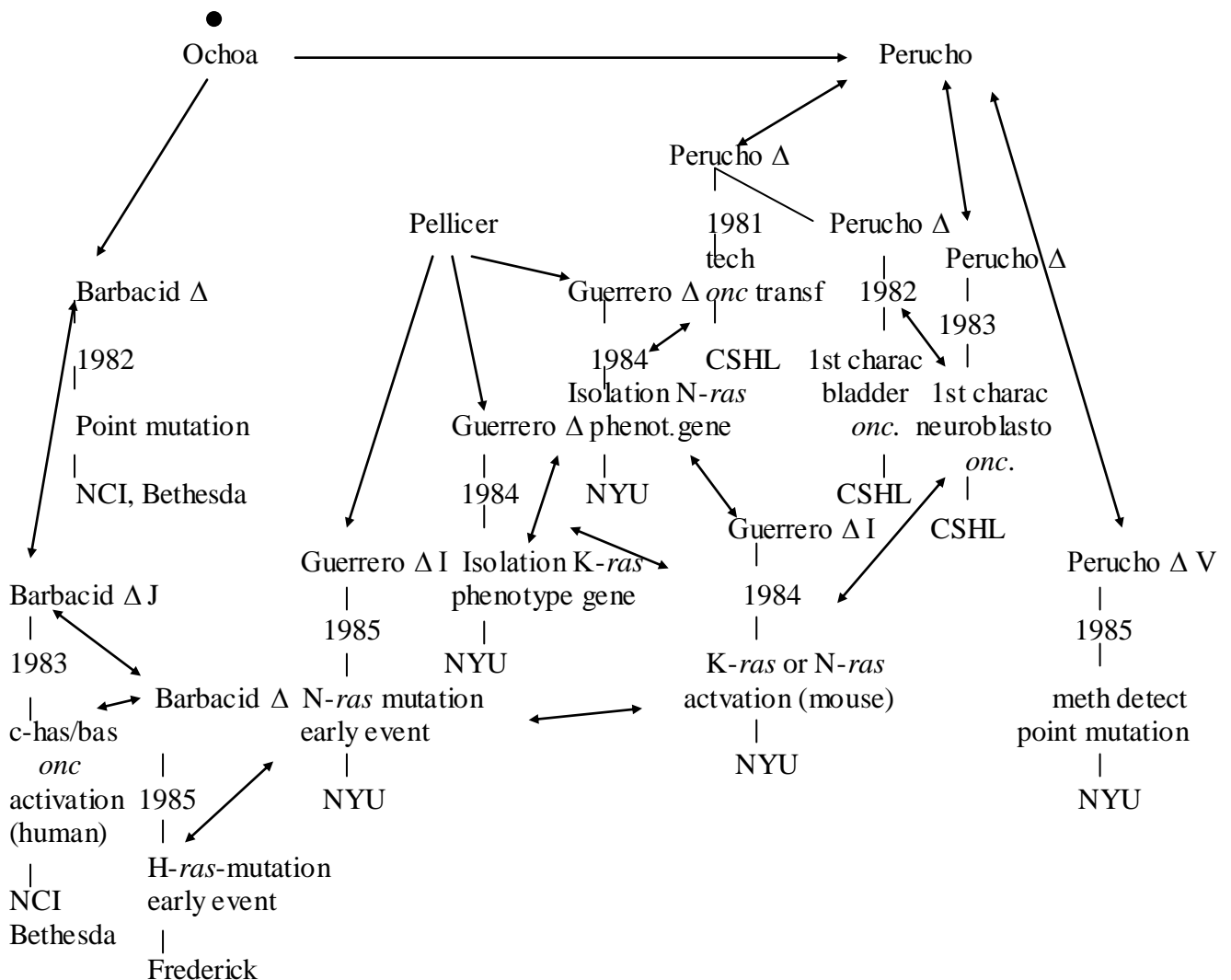
²² Villasante, A.; Corces, V.G.; Manso-Martinez, R; Avila, J. (1981) "Binding of microtubule protein to DNA and chromatin: possibility of simultaneous linkage of microtubule to nucleic-acid and assembly of the microtubule structure". Nucleic Acids Research, Vol. 9, Nr. 4, pp. 895-908.

²³ Wiche, G.; Corces, V.G.; Ávila, J. (1978) "Preferential binding of hog brain microtubule-associated proteins to mouse satellite versus bulk DNA preparations". Nature, Vol. 273, Nr. 5661, pp. 403-405.

the microtubules.²⁴ The 3T6 cell line was treated with the help in chromatin preparation of I. Guerrero.²⁵

A SYSTEMIC INTERPRETATION OF THIS SCIENTIFIC COMMUNITY.-

A strictly increasing process define the cumulative monotonicity of scientific authority in terms of single-peakedness. It is what can fix the attention on the close competition in the race for oncogenes between the three core members of this Spanish community in US, Barbacid, Pellicer and Perucho. This relation between individual careers consist of being as source authors and as cited authors continually approached by the dynamics of the scientific community.²⁶ A number of interacting processes can be advanced, each embedded in the next.



²⁴ Microtubules associated to neuroblastoma cell differentiation, otherwise useful for centromeric localization. see human artificial chromosome new gene therapy at <http://www.garfield.library.upenn.edu/histcomp/human-artificial-chromosomes/>

²⁵ Non-histone proteins in this sequence of chromosomal regions, chromatin (from *D. melanogaster* embryos), was her dissertation subject at the Autonomous University of Madrid, defended on January 1, 1981. Guerrero research program has been concerned mainly with *D. melanogaster* with the exception of the three years that she spent at NYU Dpt of Pathology.

²⁶ Price, D.J. de S. (1976) "Studies in scientometrics, part 2: The relation between source author and cited author populations". International Forum on Information and Documentation (Moscow), Vol. 1, Iss. 3, pp. 19-22.

Fig. 2. Schematic of the way in which Spanish researchers produced mirror symmetrical results in US universities and scientific organizations. Between the three research teams racing for oncogenes. V's and J's symbolize the articles that were presented by S. Ochoa to US Natl Acad. I's illustrate the track of the M. Heidelberger presented group. The small deltas represents NIH 3T3 cell cultures used to obtain cleavage. Two SCI research fronts are present, 83-1740 "Oncogenes and the genetics of human cancer; viral transforming genes and their DNA structure" (left side), and 84-4046 "Characterization of human and murine cellular oncogene" (right side). Abbreviations: CSHL Cold Spring Harbor Laboratory. NYU Department of Pathology, New York University Medical Center. NCI Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland. Frederick Developmental Oncology Section, Frederick Cancer Research Facility, Maryland.

Two research fronts are present here, 83-1740 "Oncogenes and the genetics of human cancer; viral transforming genes and their DNA structure", and 84-4046 "Characterization of human and murine cellular oncogene".

Negative differences separate the two major groups of scientists that define the population of authors inside the program. It means that the problem requiring much more authority than they have assigned, continual competitive behaviour linked their productions. Although they who do not publish so much in all these years, they are also consistent with the research program modelled. They are the "underqualified" as source authors although "fair" as cited authors. They are Guerrero and Pellicer.

On the question of how much affects individual scientists in this demography, or its constituent parts (groups of scientists), the fact is that only a woman (Guerrero) is allocated to treat the program. This knowledge must be described as an insularity that require a distortion of the original structure before its narrative can be fitted neatly into place.²⁷ Figure 2 provides a sketch of this social power process.²⁸

²⁷ Price, D.J. de S. (1981) "The development and structure of the biomedical literature". In: Warren, K.S., ed. *Coping with the biomedical literature : a primer for the scientist and the clinician*. Praeger, pp. 3-16.

²⁸ Nowakowska, M. (1984) *Theories of research*. Seaside, California, Intersystems publications. (see pp. 164-169, 'Reference frames').

CONCLUSION.-

The competing oncogene theory, of Robert Huebner and George Todaro, hypothesizing a genetic rather than an infectious mechanism for cancer, and Temin's evolutionary provirus hypothesis were within the scope of the biology performed in Spain from the early seventies when José Salas was involved during his post-doctoral fellowship at NYU, with H Green, the creator with GJ Todaro of the NIH 3T3 cell line. Also tumor virologist before 1965 had in Francesc Duran-Reynals (Barcelona, 1899 - New Haven, 1958) one of the major figures in the attempt to establish the connection between tumor virus and ordinary viruses. And so it should not be assumed that the viral-genetic concept, that develop the insertion of a viral genome into cellular one, did not exist as early as in the 1920s. It follows that when a new molecular concept of tumor growth describing cancer as dysregulation of individual genes was advanced, a unifying concept of genetic cell abnormalities as a central determinant of cancer emerged from several Spanish leaders of US laboratories in the early 80's.

To be true in terms of exogenous and endogenous factors which do trigger cellular oncogenes the new carcinogen theory worked with a cell line (NIH 3T3) with a good number of genetic alterations subjacent to neoplastic transformation. As trained in chromatin patterns Isabel Guerrero managed to be in profound understanding of 3T3 cells in Spain, through her involvement in the molecular biology of a component of the cytoskeleton, the microtubules. By the hope that in spite of the bias introduced in the analysis by the method of detection, derived from the hazardous amount of previous infection behind NIH 3T3 cells, she integrated A. Pellicer lab in the NYU, in the race for oncogenes that occurred in the mid-eighties. To work under the assumption that 10-15% of all the human tumors analyzed contained activated *ras* oncogenes. What indicated that it constituted an important biological event and that its relevance was not overemphasized by the selection of experimental systems.

Two of the main competitors were M. Barbacid, a Spanish chemist that started his virologist work with S. Aaronson, and M. Perucho who had work with M. Wigler in plasmid rescue and gene transfer.

The selective controversy surrounding the NIH 3T3 cells made them a revealing tool perceived as a frontier associated to malignant properties in vivo. In connection with signaled results from the labos of Barbacid at Bethesda and Perucho at Stony Brook, after previous successes well reported by S. Ochoa at US Natl Acad of Sci. Under the auspices of Pellicer a unique role for oncogene *N-ras* in mammals hematologic malignancies was suggested. So answering the question on if it were the cultivated cells that activated their gene after transfection while a different oncogen was operative in primary tumor cells.

By providing additional evidences in favor of *ras* gene activation in early stages, Pellicer supplied robust support to the hypothesis that *ras* mutation is the primary event in carcinogenic models. He also reported on thymic lymphomas in favor of genetic arguments linking the mutated version of *ras* genes with tumor development and against claims that *ras* genes are activated by truncation and not by point mutation. As contributing in favor of γ -radiation as a source of oncogene mutations he crucially intervened in the debate on mutation specificity for a carcinogen.

The inestimable position of Spanish biologists, in touch with NIH 3T3 cells from its inception, made them technically robust in the search for *ras* oncogenes. A medical doctor, Ángel Pellicer activated and characterized the genes involved in point mutations by using viral transforming genes obtained through transfer methods developed by him, first in Columbia University and later in the Dpt of Pathology, NYU. The responsibility for the initiation or progression of tumors, and the issues of mutation specificity have been his successful path to solve the dialectic between the chemist and the biologist in the first steps of molecular oncology.

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