Advances in CELL CULTURE

Edited by

KARL MARAMOROSCH

VOLUME 2

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PREFACE

Since 1897, when Leo Loeb took the first steps to maintain blood cells, connective, and other tissues outside the body in plasma or serum, there has been a remarkable increase in the volume of published research in the field of cell culture. There are several journals as well as numerous, excellent books devoted primarily or exclusively to this subject. Today cell culture is a scientific discipline which operates far beyond the narrow confines of its original goals.

Surprisingly, no periodical review of this field has appeared as yet, and the aim of this new serial publication, *Advances in Cell Culture,* is to fill this gap. The volumes will have international appeal, and will deal with all aspects of cell culture. "Cell culture," as used in this publication, includes the growth of individual cells or cell populations, the growth of small fragments of explanted tissue, the growth of organs, and the growth of obligate parasites in cell culture systems.

Volumes of *Advances in Cell Culture* will provide critical reviews of important aspects of *in vitro* cultivation and will reflect the increasing understanding of the wide ramifications of *in vitro* techniques. For this task we shall be relying on the continuous cooperation of our colleagues in many countries to review, synthesize, and interpret the advances made in their individual areas of investigation. It is our hope that *Advances in Cell Culture* will reveal from year to year the dedicated quest for the mastery of cell culture and the combined efforts of eminent authorities to evaluate new information so as to benefit all who use *in vitro* techniques in basic and applied research.

I am grateful to the Board of Advisors—Paul J. Chapple, Andreas Dübendorfer, Harry Eagle, Edwin H. Lennette, Toshio Murashige, Keith R. Porter, and James S. Porterfield—who will continue to suggest authors and review topics, thus providing invaluable assistance in the preparation of the volumes in this series. I am also indebted to the staff of Academic Press for their aid in producing this book.

KARL MARAMOROSCH

R. Goldschmidt.

RICHARD BENEDICT GOLDSCHMIDT
1878–1958

In the first volume of *Advances in Cell Culture* a biographical note was devoted to Ross G. Harrison, whose work formed the basis of vertebrate cell culture. In this second volume we felt it appropriate to include the biography of Richard B. Goldschmidt, the first pioneer of invertebrate tissue culture.

Goldschmidt was born in Frankfurt-am-Main, Germany to a wealthy, old German-Jewish family. He attended primary and high school in Frankfurt. His interest in biology started at 13, and by the time he was 18 he decided to become a biologist. At the request of his parents he first enrolled at Heidelberg University as a medical student, but two years later went to Munich where he became a zoology student. He returned to Heidelberg to receive his Ph.D. degree. His compulsory service in the German army ended with the withholding of an officer's commission because of his Jewish background. Similar experiences throughout his life in Germany had an impact on his personality (7). He returned to Munich University and remained there until the outbreak of World War I in 1914. During these early years he published several papers on histology, cytology, protozoology, and embryology. His most important work was on the nervous system of *Ascaris,* done from 1903 to 1910.

Goldschmidt then became interested in cell research, particularly meiosis. At the age of 29 he founded the *Archiv für Zellforschung,* and in 1911 he published one of the first textbooks on genetics. His work with the gypsy moth, *Lymantria dispar,* resulted, in his own words, in "an unbelievable piece of luck." He crossed the European moth with isolates from Japan and obtained normal males, but abnormal females that were sex intergrades. He repeated the crosses on a larger scale using other geographic isolates of gypsy moth. The results were outside the accepted laws of Mendelian genetics and formed the basis for the balance theory of sex determination.

In 1914 Goldschmidt was appointed a member of the newly created Kaiser Wilhelm Institut, and at the same time he obtained a fellowship to Japan to collect races of *Lymantria.* His return from Japan was prevented by the outbreak of the war in Europe, so Goldschmidt came to the United States and began working at the Osborn Zoological Laboratory at Yale University as a guest investigator. There he became associated with Ross G. Harrison. At that time, undoubtedly under Harrison's influence, he decided to attempt the cultivation of insect cells and thus became the pioneer of invertebrate cell culture. He suc-

ceeded in obtaining the spermatogenesis of the cecropia moth *in vitro*. He published his first paper on this subject, entitled "Some experiments on spermatogenesis *in vitro*" in 1915 in the *Proceedings of the National Academy of Sciences* (1). This contribution was followed in 1916 by a paper in German, "Notiz über einige bemerkenswerte Erscheinigungen in Gewebekulturen von Insekten" (A note about some remarkable events in the tissue culture of insects), in *Biologisches Zentralblatt* (2). The third and last paper on invertebrate tissue culture by Goldschmidt (3) was published in 1917 in *Archiv für Zellforschung*. It describes all his attempts to obtain spermatogenesis *in vitro* ("Versuche zur Spermatogenese *in vitro*"). Unfortunately, he did not follow up this work, and the next important step in invertebrate cell culture was made two decades later when William Trager succeeded in maintaining silkworm tissues *in vitro* (8) and, shortly thereafter, mosquito tissues (9), providing evidence for the multiplication of equine encephalitis virus in mosquito tissues.

J. S. Nicholas (6) recalls an incident that occurred during World War I. One night three graduate students who lived in the Osborn Tower were awakened by a Military Intelligence officer who wanted to know why lights were burning in certain rooms. The rooms were those of Goldschmidt's laboratory and the incident gave rise to rumors that he was sending signals to German submarines off the coast of New Haven. Many accepted this version even though the windows were at the rear of the Osborn Laboratory and the lights could not be seen from the harbor. When the United States entered the war, Goldschmidt was interned at Fort Oglethorpe in Georgia. After the war, he returned to Germany and continued his work in genetics.

In 1936 Goldschmidt was forced by Nazi Germany to leave the Kaiser Wilhelm Institut. He was offered a professorship at the University of California in Berkeley. He described this event as one of the happiest in his life. In 1942 he became a United States citizen and in 1947 he was elected to the National Academy of Sciences. His theory of evolution and his outdated concepts of genes and alleles were all but appreciated by modern geneticists, but in 1951 he was invited to deliver the opening address at the Cold Spring Harbor Symposium on Chromosomes and Genes (4). It was my good fortune to attend this symposium at the suggestion of Keith R. Porter and George E. Palade. At that time I knew nothing about Goldschmidt's pioneering tissue work, but his name was well known to me from my genetics course. During the symposium he was interested mainly in the work of molecular geneticists, particularly in the results presented by Barbara McClintock.

In September 1953 I saw Goldschmidt for the last time at the 9th International Congress of Genetics in Bellagio, Italy, where he was honored as the President of the Congress. Shortly thereafter he suffered a severe heart attack. Despite his illness, he was able to write an extensive treatise, "Theoretical Genetics" (5), many parts of which remain relevant to current genetic work.

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CELLULAR SENESCENCE *IN VITRO*

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I. Introduction: Definitions of Aging

Biological aging may be defined as the sum of all time-related change occurring in an organism throughout its life span. In general, the term "senescence" includes those changes occurring in the postreproductive period that are progressively deleterious, intrinsic,

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and common to all members of a species (Strehler, 1977). Some of these criteria are easier to defend than others. For example, it is clear that senescence must be at least partly under genetic control because maximal life span is a species characteristic. However, it is also clear that environmental events influence life span. Although all members of a population with a finite life span senesce, individuals within that population exhibit the changes associated with senescence at somewhat different chronologic ages. The role of the environment in the expression of these changes remains unclear. Much of the confusion in definitions of aging stems from the fact that death is used as an endpoint for aging. Although death is an obvious and convenient endpoint, it can, however, be totally misleading as it may or may not be the result of changes that occur during aging.

The senescence of mammalian fibroblast-like cells *in vitro* may be characterized most simply as a decrease in the proliferative capacity of populations of these cells with time. Cells which undergo *in vitro* senescence, in contrast to "immortal" transformed lines, maintain an essentially normal (diploid) karyotype in culture and exhibit a finite life span which, for those species studied, appears to be related to the life span of the species of origin and the donor tissue. But even for cells *in vitro* questions remain. For example, is senescence intrinsic or extrinsic to the cells? If intrinsic, what is the nature of the regulatory mechanism? And does aging *in vitro* reflect aging *in vivo?*

II. Historical Background

A. Limited Replicative Life Span of Cells in Culture

Whether or not senescence is a supracellular phenomenon is a question that has been asked by many scholars over many decades. An early idea proposed by Weismann, Minot, and other biologists, stressed the possibility that aging might be the price for cellular differentiation, and that cells removed from the constraints of the animal body might be immortal. Alexis Carrel and A. H. Ebeling believed that they had demonstrated immortality in isolated cells by keeping cultures of chick heart fibroblasts alive and proliferating for 34 years, a period longer than the chick life span, before voluntarily terminating their experiment (Carrel, 1912, 1914, 1935; Ebeling, 1913).

Indirect support for Carrel's observations came from the discovery of a number of cell lines, including "L" cells derived from mouse mesenchyme (Earle, 1943) and HeLa cells derived from a human cervical

carcinoma (Gey *et al.*, 1952), which could be grown continuously in culture without a decline in proliferative vigor. Thus, the notion that "cells were immortal, organisms mortal" seemed reasonable. It was not until 1957, when Swim and Parker reported a limited life span for human embryonic and adult tissue fibroblasts in culture, that there was an indication that cells, like the parent organism, might age. In 1961, Hayflick and Moorhead observed in a large study that cells from a variety of normal human tissues proliferated in culture for various periods of time but eventually degenerated and died. After outgrowth from the explant, a period of rapid multiplication followed during which frequent subcultivations were possible. Later, the proliferative capacity of the cells decreased, intracellular debris accumulated, nuclear abnormalities appeared, and the culture died. These workers later proposed that the limited replicative potential of human cells *in vitro* was an expression of senescence at the cellular level (Hayflick, 1965). Hayflick's observations were confirmed by Hayakawa (1969) and subsequently by many workers for cells from a variety of tissues and organisms.

By contrast, attempts to reproduce Carrel's findings have not been successful. Even when techniques similar to Carrel's were used (Gey *et al.*, 1974), chick cultures could not be maintained for more than 44 months. Hayflick (1970, 1975, 1977) has suggested that Carrel, in preparing his chick embryo extract supplement to the culture medium, may have introduced new viable cells at each refeeding. All of the other cell lines that reportedly have an indefinite life span have been shown to express at least one of the characteristics of transformed cells.

Further support for the Hayflick view has come from the findings of several workers who have shown that aging *in vivo* is reflected in cell culture. A number of workers showed that the time between introduction of embryo tissue into culture and cell migration from the explant increased with age of the embryo (Carrel and Burrows, 1910, 1911; Cohn and Murray, 1925; Suzuki, 1926; Hoffman *et al.*, 1937; Medawar, 1940; Lefford, 1964). Also, many workers (Soukupova and Holeckova, 1964; Soukupova *et al.*, 1970; Waters and Walford, 1970; Soukupova and Hnevkovsky, 1972) have reported that the outgrowth of cells, particularly fibroblasts, from rat and human tissue explants decreased with increasing age of the donor. In 1975, Ooka *et al.* reported a similar decrease in rate of migration of rat epidermal cells in culture with age of donor. Hayflick (1965) additionally found that when mixtures of young and old populations of human fetal lung fibroblasts were grown together in the same culture vessel, the older population died first at

the same number of population doublings as "old" matched controls, while the young populations continued to grow vigorously until reaching approximately the same number of total population doublings. At this time they also died. This experiment seemed to rule out the possibility that life span limitation might be caused by deficiencies in the medium, microbial contamination, or toxic wastes produced by the cells and indicated that it was, instead, intrinsic to cells. Additionally, when populations of cells were frozen in liquid nitrogen at a certain population doubling level and later thawed and recultured, the cells continued to divide until they had reached the average total number of doublings characteristic of that line (Hayflick, 1965). These experiments collectively indicated that senescence in diploid cells *in vitro* seemed to be intrinsic to the cells and precisely timed.

B. In Vivo Correlations

Normal diploid cells provide a useful model system for the study of aging because of their finite life span in culture. It is possible, for example, to compare the proliferative capacities of cells grown *in vitro* from donors of different ages. Hayflick (1965) showed that an average of 20 (14–29) doublings occurred in adult human lung fibroblasts whereas an average of 48 (35–63) occurred in cells from human embryos. In 1969, Goldstein *et al.* reported an inverse correlation between the age of the donor and the number of population doublings attained by cells from a series of skin cultures. Martin and co-workers (Martin *et al.*, 1970; Martin, 1977) confirmed Hayflick's original observation by culturing fibroblasts from biopsies of upper arm tissue taken from human donors ranging from embryos to persons 90 years of age. A significant correlation between life span and donor age was found; the older the donor, the shorter the life span of the derived cells in culture. Extensive studies by Schneider and Mitsui (1976) of the *in vitro* growth of upper arm skin fibroblasts from old and young human donors have shown a significant decrease in the rate of fibroblast migration, length of *in vitro* life span, rate of cell replication, and cell number at stationary phase when cells from old donors (63–92 years) were compared to cells from young donors (21–36 years). No change was found in cell volume or macromolecular content. Percentage replicating cells and percentage of cells able to form colonies of more than 16 cells also decreased with increasing donor age (Schneider, 1979). The differences between growth of cells from young and old donors *in vitro*, while significant, were not as large as the differences found in a parallel study between early and late passage human fetal lung (WI-38) fibro-

blasts (Schneider and Mitsui, 1976; Schneider *et al.*, 1977a). Although these differences were attributed to the different tissues of origin (skin and lung), it is possible that the discrepancy reflects different lengths of time spent in culture since the cells from both groups of adult donors in the 1976 study were only followed for 10 population doublings in culture whereas WI-38 cells were compared at 19 and 39 doublings. Fetal human lung fibroblasts, in comparison to fibroblasts from fetal human skin, exhibited a faster replication rate, a 2- to 5-fold greater incorporation of [^{3}H]thymidine into DNA, 3 times as many cells at confluency, and a longer life span by about 10 population doublings. Lung cells had a smaller volume and contained less RNA and protein. Attachment and percentage of nuclear labeling after the first 12 hours were equal. Thus, *in vitro* manifestations of *in vivo* age changes occur, but tissue-specific differences may also affect the *in vitro* life span of cells. Alternatively, skin fibroblasts may undergo more replications *in vivo,* even in the embryo, and hence will have completed more of their population life span than lung cells at the time of removal from the host. Recently, papillary fibroblasts have been found to proliferate *in vitro* more readily than reticular fibroblasts from the same skin specimen (Harper and Grove, 1979). Thus, samples from different tissues may yield different types of fibroblasts with different life spans. Martin *et al.* (1970) had previously found that skin cells live longer in culture than bone marrow cells and that cells from skeletal muscle live for an intermediate length of time. LeGuilly *et al.* (1973) have suggested that the multiplication potential of cells in tissue is inversely related to degree of differentiation.

An inverse relationship between growth potential and increasing age of the donor has been found for tortoise fibroblasts (Goldstein, 1974; Macieira-Coelho, 1976) and for mouse aorta and adventitia (Martin *et al.*, 1975a). It has been suggested that the population doubling maxima of normal embryonic fibroblasts *in vitro* are proportional to the mean maximal life span of the donor species (Hayflick, 1970, 1975, 1976, 1977; Sacher and Hart, 1977; Rohme, 1981), although this relationship may not hold true for other cell types nor for cells *in vivo* (Cameron, 1972a,b; Hayflick, 1975). Table I summarizes the available data for those species that have been studied.

A recent finding by Ryan *et al.* (1981) supports the concept that cell life spans are genetically determined. They found that skin fibroblasts from three pairs of monozygotic twins showed no significant difference in replicative life span within each twin pair, but did show such differences among pairs.

Decreased replicative capacity with increasing donor age has also