In Vitro Cell. Dev. Biol. 26:9-23, January 1990

0883-8364/90 $01.50+0.00

Invited Review

FRONTIERS IN MAMMALIAN CELL CULTURE*

WALLACE L. Mc KEEHAN, DAVID BARNES, LOLA REID, ERIC STANBRIDGE, HIROKI MURAKAMI, AND GORDON H. SATO

W. Alton Jones Cell Science Center, Inc., 10 Old Barn Road, Lake Placid, New York 12946, (W. L. McK., G. H. S.); Department of Biochemistry and Biophysics, Environmental Health Sciences Center, Oregon State University, Corvallis, Oregon 97331 (D. B.); Departments of Molecular Pharmacology, Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461 (L. R.); Department of Microbiology and Molecular Genetics, University of California, Irvine, Irvine, California 92727 (E. S.); and Graduate School of Genetic Resources Technology, Kyushu University, Fukuoka 812, Japan (H. M.)

(Received 1 September 1989; accepted 2 October 1989)

SUMMARY

For the past 60 years, fundamental discoveries in eukaryotic biology using mammalian cell cultures have been significant but modest relative to the enormous potential. Combined with advances in technologies of cell and molecular biology, mammalian cell culture technology is becoming a major, if not essential tool, for fundamental discovery in eukaryotic biology. Reconstruction of the milieu for cells has progressed from simple salt solutions supporting brief survival of tissues outside the body to synthesis of the complete set of structurally defined nutrients, hormones and elements of the extracellular matrix needed to reconstruct complex tissues from cells. The isolation of specific cell types in completely defined environments reveals the true complexity of the mammalian cell and its environment as a dynamic interactive physiological unit. Cell cultures provide the tool for detection and dissection of the mechanism of action of cellular regulators and the genes that determine individual aspects of cell behavior. The technology underpins advances in virology, somatic cell genetics, endocrinology, carcinogenesis, toxicology, pharmacology, hematopoiesis and immunology, and is becoming a major tool in developmental biology, complex tissue physiology and production of unique mammalian cell-derived biologicals in industry.

Key words: biotechnology; cell biology; cell cloning; cell nutrition; defined cell culture; differentiation; extracellular matrix; molecular biology; oncogene; retrovirology; somatic cell genetics; virology.

CELL CULTURE RESEARCH

Animal cell culture is rooted in two early concepts in biology. Schleiden and Schwann (1,2) postulated the cell as the fundamental unit of life and the possibility of its autonomous existence. Claude Bernard's concept (3) of homeostasis and the constant internal milieu within tissues presented a rationale and the stimulus to undertake the culture of animal cells. Mammalian cell culture research is a two-way study of the cell and its environment, although each facet has been the subject of empirical study without regard for the other.

Major goals of cell culture research are: (i) to isolate specific mammalian cells that retain specific properties observed in vivo; (ii) to characterize and determine mechanism of action of the environmental factors that directly affect cell behavior; (iii) to reconstruct the cell and its environment into a defined, experimentally manageable unit for study of determinants of responses and properties of cells in a dynamic context; and (iv) to reconstruct cells and their environment into tissues and organs.

Classical cell culture technology is carried out in nutrient mixtures supplemented with a biological fluid, most commonly serum. Culture of tissue cells usually involves a monolayer of cells attached to hydrophilic surfaces. The technology evolved from application of microbiological methods to isolate, grow and observe cells from embryonic or connective tissues in fluids from the embryo or wounds (4-6). Primary cultures are cultures prepared directly from tissue. Serial cultures are prepared by serial harvest, transfer and dilution of cells from primary cultures or stocks of cells stored under cryoprotective conditions. Technical innovations re-

*This article is the first of a series of invited reviews aimed at identifying fundamental contributions and current challenges associated with research activities in subdisciplines of cell and developmental biology in vitro. This treatise is dedicated to Dr. Brian Kimes, Program Director at the National Cancer Institute, whose vision, encouragement and support have contributed significantly to modern developments in mammalian cell culture.

quired to observe living cells, to sterilize cell culture reagents, and to maximize the number of cells that can be grown in a minimum amount of medium have emerged from diverse research efforts, but the collective technical and conceptual contributions of George and Margaret Gey are worthy of special note (7-12). The Geys were the first to demonstrate the growth effects of a hormone (insulin) (7), that extracellular matrix components (collagen) enhance cell attachment (12), and the production of a hormone (gonadotropin) by cells in culture (10). The Geys established the first and most widely used model human tumor cell line (HeLa) (11). Acquiring the capacity for proliferation indefinitely in culture, a major advantage to the experimenter, is a rare event for normal tissue cells from most species except rodents (13-15). Cultured cells, and cell lines in particular, bear the caveat that they rarely exhibit the full spectrum of properties exhibited by normal tissue cells. Skepticism about the fidelity of individual cell cultures to cells in tissues is intrinsic to the design of hypotheses to be tested. The homogeneity, reproducibility and amount of material offered by cell cultures and individual cell lines in particular, when properly applied as a basic research tool, has yielded basic and generally significant discoveries to eukaryotic biology to neutralize skepticism about fidelity to tissue of origin (Table 1). Although specific cell culture conditions and some properties of specific cultured cells may be artificial, basic mechanisms revealed by study of selected aspects of cultured cells can rarely be challenged as artifact that have no physiological basis.

Defined cell culture environments. The development of a defined cell culture environment commonly referred to as "serum-free, defined medium" or "hormonally-defined medium" is a long-term goal of the cell culturist (31,39-48). Successful applications have largely evolved by synthesis of the most current advances in isolation and characterization of individual nutrients, hormones/growth factors, and elements of the extracellular matrix from tissues and fluids into a complete medium that minimizes or eliminates the requirement for serum or other undefined biological fluids and extracts (39-48). Despite advances in high performance purification and analytical methods, direct approaches to isolate and characterize all the active principles in serum or alternative biological fluids using a specific cell culture response as an assay have largely been defeated by the low concentration and multiplicity of bioactive components in crude biological fluids and the endogenous production of similar factors by cells. The analytical approach has been most effective when the number of active factors in undefined additives can be reduced by the synthetic or replacement approach to one or a few factors on which the cell culture is dependent. Development of a completely defined environment for normal prostate epithelial cells is an example of systematic application of a combined synthetic and analytical approach (56-59). First, a semi-automated replicate growth assay was established and libraries of defined growth promoters (nutrients, hormones/growth factors, matrix factors), undefined tissue extracts, and the medium of diverse cultured cells were screened for stimulation in medium containing growth-limiting levels of serum (56). Neural extract (hypothalamus, pituitary, spinal cord) was found to be the most active tissue extract or fluid. Second, the nutrient medium was optimized to reduce requirement for serum and neural extract to a minimum (56). Third, the screening of defined hormones and growth factors resulted in synthesis of a mixture which eliminated the serum requirement and minimized the requirement for neural extract (56). The active factor from neural extract, a polypeptide in this case, was isolated and utilized to replace crude neural extract and to complete the synthesis of the defined medium (58,59).

Robust and exhaustive methods employing combinations of the synthetic and analytical approach to development of defined cell culture environments have been applied to a wide variety of cell types and applications and have led to several generalizations (40-48,60-66): (i) many cell types require a quantitatively balanced set of common nutrients and cell-type specific nutrients (60-63); (ii) cells are dependent on multiple hormones and elements of extracellular matrix that act synergistically to regulate both growth and expression of function (40-48,64-66); (iii) many cell regulators, both soluble factors (40-48,63,67) and fixed components of the extracellular matrix (64-66), are produced by cells in the local cell and tissue environment; (iv) most cells respond to insulin (or insulin-like factors), the iron-transferrin complex, and one or more lipids complexed to a suitable carrier, such as fatty acids/albumin or natural lipoproteins (41-48); (v) most mesenchymal cells (except endothelial) respond to platelet-derived growth factor (PDGF) or a related factor (68,69); (vi) many normal epithelial cells respond to an agonist of cyclic AMP metabolism such as cholera toxin (56,70,71); (vii) the factors required for tumor cell growth and many continuous cell lines are often a subset of requirements for the normal cell counterpart (40-48,56-59); (viii) a specific medium can select for cell type and stabilize cell-specific mRNAs (72,73); (ix) serum and other crude biological fluids and extracts contain inhibitors of

TABLE 1

EXAMPLES OF DISCOVERIES USING CULTURED MAMMALIAN CELL LINES

Discovery	References
Macromolecule processing	16-20
Mechanism actinomycin D	21
Human karyotype	22-26
Fluidity of membrane antigens	27
Viral transformation	28-30
Polypeptide growth factors	31,32
Lymphokines/interleukins/cytokines	33-38
Complex cellular endocrine physiology revealed through defined media	31,39-48
Retrovirology	49
Hybridomas	50
Somatic basis of immune gene diversity	51
Tyrosine kinases	52
Oncogenes/proto-oncogenes	49,53-55

tissue-specific cells (74,75) and are enriched in stimulators of mesenchymal cell growth (68,69); and (x) an individual hormone (74–78) or matrix factor (64–66) may act as agonist or antagonist of cell growth or function dependent on cell type and context of assay. Successful application of the technique to support expression of various properties of virtually any cell type capable of expressing the property in vivo appears to be limited only by (i) the large catalog of candidate factors that must be systematically screened and synthesized into a complete medium and, (ii) the identification, isolation and chemical characterization of novel, uncharacterized factors from extracts of tissues, the fluids from the tissues or from cultured cells that exhibit the desired cellular properties.

Completely defined cell culture environments achieve chemical definition, standardization, reproducibility and investigator-control that is desireable for practical applications. Defined medium can be rationally designed to select for growth or function of specific cell types or practically any individual aspect of cell behavior.

The concepts that have emerged by application of the technique challenge views of the isolated mammalian cell as a microorganism (79) and the classical endocrinological concept of target cell regulation by one or a few specific trophic hormones (31,40–48). The technique has revealed the true complexity of the cell and its environment as a dynamic interactive system or physiological unit. The isolation of every animal cell type and complete definition of its environment combined with modern molecular biology promises to allow step-by-step dissection of the mechanisms that determine mammalian cell behavior in its dynamic physiological context and eventual reconstruction of tissues and organisms.

Cell nutrition. Serum and other heterogenous biological fluids contain nutrients that support the growth and function of cultured cells. By using serum from which low molecular weight components were removed by dialysis, the requirement for the dietary nonessential amino acid, glutamine was demonstrated (80). Empirically designed mixtures, nutrients and other biochemicals made available from the work of nutritionists and biochemists made large contributions to characterization and replacement of the nutritional role of serum and other biological fluids in cell cultures (81–86). Eagle applied a systematic, biochemical analysis of requirement and metabolism of nutrients in cell cultures containing dialyzed serum (87,88). Variants of the minimal mixture of salts, amino acids, vitamins and carbohydrates that emerged have been and are still widely employed and were the precursor to the more complete, rigorously balanced, nutrient mixtures designed for specific cell types and defined cell culture environments (60–63).

The development of the optimal nutritional environment for different cell types and applications is often critical for characterization and synthesis of the complete defined environment of specific cell types (60–63). These activities have contributed basic findings and concepts (61–63) such as (i) the requirement of human cells for selenium and other trace nutrients (89); (ii) the requirement of some cells for phospholipids and ethanolamine (90–93); (iii) the individuality of the optimal nutritive environment required by specific cells (91–93); (iv) the informational content of specific nutrients (94); (v) the regulation of cellular nutrient requirements and metabolism by specific hormones (62,95,96); (vi) the role of glutaminolysis in cell proliferation (97,98); and (vii) the reduced nutrient requirements of tumor and transformed cells (62,99).

Novel polypeptide hormones. The lack of response of most cultured cells in serum and other biological fluids to individual hormones elucidated by conventional animal endocrinology was a major obstacle to application of cell cultures to study physiological regulators and their mechanism of action (31). The negative results were interpreted as (i) a failure of conventional cell culture conditions to support the target cell type and its responsiveness to trophic hormones; and (ii) the presence of multiple bioactive factors in serum or other biological fluids that masked responsiveness to single hormones. Growth and other phenotypic properties of cultured cells from endocrine target tissues could not be supported by a single trophic hormone (31,39–48). However, an array of partially- or chemically-defined polypeptides from diverse fluids and organ extracts stimulated cells when serum supplements were reduced or depleted by specific treatments. This led to the realization that cells required complexes of conventional hormones and novel hormone-like polypeptides present in serum at low levels or missing from serum altogether (31,40,41). Since the first serum-free, hormonally-defined medium was synthesized from structurally characterized hormones for a pituitary tumor cell line (39), cell cultures, especially in serum-free media, have become the tool for discovery (i) that some classical hormone effects on growth in vivo are mediated by novel, direct-acting polypeptides in serum and tissue extracts (100–103); (ii) that the blood platelet is a rich source of hormones that are released into serum during clotting (68,69,74,75); and (iii) that hormone-like polypeptides originate and act directly on cells in local tissues (32,67). Cell cultures have resulted in discovery, classification, and characterization of biological activities and, to some extent, mechanisms of action of at least 10 families of polypeptide hormones (32–38,68,69,74,75,101–106). These polypeptide hormone-like regulators of cell behavior cluster into families consisting of sometimes up to 7 gene products (105) that have either similar biological effect, structural homology, or homólogous receptors.

Extracellular matrix. Until the late 1970's, the extracellular matrix, which is a mixture of collagens, noncollagenous proteins, and carbohydrate-rich molecules was considered a static, structural support for cells in vivo. More recently, cell culture assays have revealed that the matrix consists of distinct molecules that affect cell behavior (64–66,107–111). Differentiated cells can be induced to grow or locked in a state of growth arrest by varying components of the extracellular matrix (64–66). Although purified matrix components such as collagens, adhesion glycoproteins (fibronectin, serum-spreading factor/vitronectin, laminin, etc.) and proteoglycans affect tissue-specific functions, the optimal responses have been made with tissue extracts enriched in extracellular matrix (112–115).

Study of the factors which mediate adhesion of cultured cells to substrates has revealed that many

molecules that mediate cell adhesion contain a common cell recognition sequence (arginine-glycine-aspartate) and are recognized by receptor-like molecules (integrins) that span cell membranes (116,117). The integrins are thought to link extracellular matrix factors to the cytoskeleton, and may generate signals similar to receptors for soluble molecules that determine cell behavior. Specific components of the extracellular matrix also directly interact with and determine the activity and stability of soluble polypeptide regulators such as heparin-binding (fibroblast) growth factors whose direct action is mediated by cell membrane-bound receptors distinct from the extracellular matrix (105,106). Hormone-like polypeptides modify the composition of the extracellular matrix and the expression of matrix factor receptor sites (118-121). A major challenge remains to determine (i) which effects of extracellular matrix are mediated by matrix-regulated polypeptide hormones; (ii) which effects are a direct consequence of matrix factor-receptor interaction; and (iii) what is the signal generated by matrix factor-receptor interaction.

Cell cloning. Single cell (clonal) culture techniques (122-127) are indispensable for isolation and selection of pure populations of mutant cell lines, cell hybrids, specific cell types direct from tissue (128-132), and quantitative analysis of the actual nutritive and hormonal requirements of cells without interference by cell-derived products (60-63). Development of clonal culture methods, considerably more challenging than development of methods for cultures of higher density populations, has revealed the extreme dependence of cell behavior on composition of and the extent to which populations of cells modify the microenvironment (60-63). The cloning of animal cells was first achieved by placing single mouse fibroblasts in capillary tubes which were immersed in a large volume of medium (133). The medium to cell ratio (v/v) in the tube was sufficiently small so that cells could modify the medium in the tube and then proliferate. Cells that migrated into the larger culture vessel died unless the medium from a mass culture of cells was added. The use of medium from or co-culture with dense populations of homologous or heterologous cell types (71,122) often facilitates single cell culture until the principles of defined cell culture environments can be rigorously applied to support the isolated single cells.

Differentiated cells in culture. Ross Harrison, who demonstrated the growth of nerve cell processes from fragments of spinal ganglia embedded in clotted lymph in 1910 and who is credited with the first significant application of tissue culture, used the technique to answer a question about specific differentiated tissue (134,135). For more than fifty years, cell cultures which performed the specialized functions of differentiated normal and tumor tissues were not forthcoming. Tissue culture cell populations were thought to either be descendants of tissue-specific cells which lost differentiated properties in the process of multiplication in culture or ubiquitous mesenchymal cell types (fibroblasts or vascular cells) common to different tissues (136). Direct cloning of cells from suspensions of disaggregated tissues and primary cell cultures indicated the presence of cells

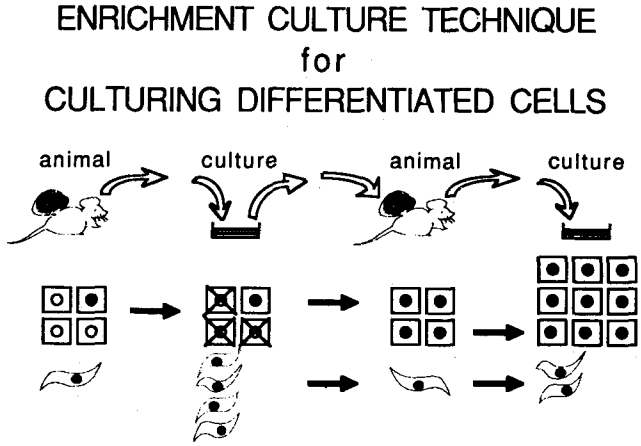

FIG. 1. Transplantable, differentiated tumors consist of a few differentiated parenchymal cells (squares/closed circles) with sufficiently relaxed growth requirements to survive among the abundant spindle-shaped fibroblast-like cells in classical serum-containing primary cultures. Introduction of the cells from primary culture back into the host gradually amplifies the ratio of the immortal, highly tumorigenic, differentiated parenchymal tumor cells to the mortal, non-tumorigenic host-derived fibroblast-like cells (142-148).

whose tissue-specific functions could be manipulated (128-132). However, efforts to grow, maintain and manipulate function of mass cultures of differentiated cells in sufficient quantity for biochemical analysis were disappointing. The difficulty was overcome by a systematic in vitro/in vivo method of establishing differentiated cell cultures from well-differentiated transplantable tumors (Fig. 1) or, in a few rare cases, by differentiated cell lines that arose spontaneously (137-141). The technique has generated model functional cell lines representative of differentiated cells from neurons, adrenal cortex, glia, pituitary, interstitium, melanocytes, chondrocytes, muscle, epidermis and other tissues (142-148). The technique is applicable to practically any tissue for which a differentiated transplantable tumor model is available and where the need for large quantities of reproducible, homogenous research material offsets the limitations of the specific cell lines.

Application of the principles of defined cell culture environments to isolation, selection and culture of cells from differentiated tissues has revealed that composition of the microenvironment (nutrients, hormones, extracellular matrix) is the key determinant of the relative kinetics of growth and expression of function in populations of cells from differentiated tissues (40-48,64-66). The design of the cell culture environment to manipulate growth and expression of function of tissue-specific cells is determined by the type of tissues that are the object of study. The relatively undifferentiated proliferative ("stem") cells from constantly regenerating tissues such as skin, bone marrow, and intestine can be maintained for sustained periods before terminal differentiation occurs concurrent with loss of cell viability and growth potential (64-66). The challenge has been to determine the factors that both delay, direct and promote different lineages of cells toward terminal differentiation. The predominant functional cell types from tissues such as

liver, lung, pancreas, kidney and mammary gland can be maintained for months and undergo limited cell divisions (64-66). The challenge for these type of tissues is to synthesize conditions required to select for and sustain the proliferative cell populations and determine whether they arise from the fully differentiated tissue cells or from minor populations of undifferentiated "stem" cells or both. Defined environments that support pure proliferative populations of epithelial cells from tissues such as prostate (56-59) and mammary glands (149) have been achieved. However, conditions that will support cycling of synchronized cell populations between growth and the fully functional state from tissues which are normally quiescent, but have remarkable capacity to proliferate under certain conditions in vivo, remains a challenge. Additional challenges remain to identify environments that maintain the fully functional state of cells from tissues such as adult central nervous system and muscle which are thought to be terminally differentiated in the functional state and for which the regenerative capacity in vivo appears limited.

Detection of the Genes That Determine Cell Behavior

In addition to growth requirements, individual characteristics of cells in culture such as morphology, density-dependence, motility, adhesiveness, mortality/immortality, and tumorigenicity in host animals can be manipulated, selected for and quantitated in cultured cells and correlated with the behavior of cells in vivo during embryonic development, wound healing and cancer (13-15,28-30,150-159). Modification of these characteristics by transfer and expression in cultured cell lines of RNA and DNA viruses, artificial constructions of various genes linked to viral transcriptional/translational promoters, and most recently, pieces of genomic DNA from tumors have resulted in remarkable insight into the nature of the genes that affect individual aspects of cell behavior (53-55). The term "oncogene" describes a gene from a virus or tumor that, when introduced into and expressed in certain cultured cell lines, induces one or more of the individual behaviors associated with cancer cells, most commonly reduced adhesiveness. A "proto-oncogene" refers to a similar gene from normal cells whose oncogenic counterpart probably arose by somatic mutation.

The notion that hormone-like polypeptide regulators of cultured cell behavior are legitimate cellular regulators in vivo was solidified with the finding that some oncogenes are altered or abnormally expressed growth factor/receptor genes (53-55,160-163), and that some genes that are activated by polypeptide growth factors (53-55, 164,165) are oncogenes/proto-oncogenes. In both cases, constitutive expression of the oncogene/proto-oncogene by-passes the requirement for exogenous growth factor. The abnormal activation or over-expression of growth factor genes and gene products that are elicited by growth factors explains in part the common observation that the growth requirement for serum, other biological fluids, or specific polypeptides for cultured tumor-derived or artificially transformed cells is reduced relative to their normal or untransformed counterparts (40-48,67). Since polypeptide growth factors are metabolized by cells (see *Endocrinology* section), the findings also explain the phenomena that normal cultured cell growth, unlike transformed cells, is inversely proportional to cell density (155,157,158). Assays that detect reduced adhesiveness of selected cell lines have generated a monumental list of candidate oncogenes/proto-oncogenes, and resulted in discovery of the epidermal growth factor (EGF) homolog, transforming growth factor alpha (TGF-α) (166,167), and the transforming growth factor beta (TGF-β) family of cellular regulators (74,75). Elucidation of the mechanism of oncogene/proto-oncogene-dependent alteration in cell adhesiveness and other individual aspects of cultured cell behavior remains a major challenge.

Cultured cells are the tool for identification of the genes transiently or permanently activated by individual agents in the local tissue environment (165,168,169) and the hosts to test the phenotypic consequence of expression of practically any cloned gene or piece of nucleic acid in mammalian cells (49). As the technology for culture and manipulation of growth and function of tissue-specific cell types emerges, cultured cells promise to be the means for characterization of the genes that regulate tissue-specific functions and the process of differentiation.

Cell Culture and Traditional Disciplines of Eukaryotic Biology

Virology. Without cell culture, the major advances resulting from study of eukaryotic viruses would not have occurred (28-30,49,53-55). Development of suitable cell cultures to routinely assay and reproduce the viral life and pathogenic cycle of eukaryotic viruses such as hepatitis, influenza and immunodeficiency viruses remains a challenge.

Somatic cell genetics. The technique of somatic cell fusion using cultured cells has revolutionized human gene mapping. Cells of the same or different species fuse either spontaneously or with the aid of a fusogen, such as inactivated Sendai virus or polyethylene glycol, to form heterokaryons and stable hybrid cells containing chromosomes from both parent cells (170-172). Interspecific hybrid cells, generated from the fusion of rodent cells with normal human diploid fibroblasts or lymphocytes, rapidly lose human chromosomes. The random chromosome loss makes it possible to obtain hybrid cells that retain one or more human chromosomes. Human x rodent hybrid cell panels have been established and can be used to map virtually any human gene, either by using DNA probes that correspond to specific human genes or by detection of the human gene product (173,174). Prior to this advance, only a few human genes had been mapped by conventional genetic linkage analysis. Literally hundreds of genes have now been mapped using a combination of somatic hybrid cell panels and in situ hybridization. Human x rodent somatic cell hybrids are inherently unstable. The human chromosomes are lost by chromosomal nondisjunction during cell division or by translocations which occur either between human

chromosomes or between human and rodent chromosomes. Therefore, the somatic cell hybrid panels must be regularly checked for cytogenetic confirmation of the identity and integrity of the human chromosomes within each clone.

The technique of microcell transfer was developed whereby single human chromosomes could be introduced into rodent cells (175,176). Microcells are cell-like structures containing only one or a few chromosomes surrounded by a nuclear membrane and some cytoplasm and a plasma membrane. The difference between this technique and whole cell fusion is that only one or a few chromosomes are transferred via microcell fusion. As with whole cell fusions, the transferred chromosome is retained in the recipient cell in succeeding generations as a complete structural unit. Transfer and selective retention of individual specific human chromosomes has been accomplished by integration of dominant selectable markers into specific human chromosomes using DNA transfection or retroviral infection, followed by transfer of the chromosome containing the integrated marker gene to recipient cells via microcell fusion (177,178). A panel of microcell hybrid cell clones representing the entire human karyotype is approaching completion (E. Stanbridge, unpublished). Such a panel will facilitate unequivocal mapping of human genes to their correct chromosomal location and eventually provide a valuable resource for mapping and sequencing of the entire human genome.

Human somatic cell hybrids established the existence of tumor suppression genes (179–185). There is now mounting evidence that the multi-step progression from a normal cell to a cancerous cell involves both the activation of oncogenes and the loss of function of tumor suppressor genes, the latter being facilitated in part by chromosomal deletions (184–187). Restoration of deleted genetic information in human cancer cells and subsequent suppression of tumor formation has been accomplished by microcell transfer of single specific human chromosomes from normal cells into the cancer cells (186,187). In addition to providing direct evidence for the existence of genes which can suppress tumor formation, the microcell transfer technique allows one to pinpoint the chromosome region in which the putative tumor suppressor gene maps and to clone the gene(s) involved.

Early studies showed that fusion of a differentiated cell with an undifferentiated cell often resulted in the extinction of expression of genes involved in the control of differentiation functions. However, there are exceptions and the relative ploidies of each parental genome in somatic cell hybrids seem to be influential in determining expression or extinction of genes critical for differentiated function (188–190). Expression of previously dormant differentiation-specific genes in the undifferentiated parent cell genome is occasionally seen in somatic cell hybrids derived from the fusion between differentiated and undifferentiated parental cells (191,192). The analysis of regulation of tissue-specific gene expression using such somatic cell hybrids is in its infancy. Monochromosome transfer technology also promises to play a role in elucidation of this complex genetic interplay (193).

Endocrinology. Cell culture technology has contributed to endocrinology by (i) the development of hormone-producing and hormone-responsive cell lines in sufficient quantity for biochemical analysis (136,142–148); (ii) the methodology for mass culture of tissue-specific cells from normal endocrine tissue (40–48); and (iii) the revelation that most cells secrete novel hormone-like polypeptides that act directly on cells in the local tissue environment in an autocrine or paracrine mode (31, 40–48, 67–69).

The biochemical mechanisms of polypeptide hormone action have been largely established in cell culture. Cohen's work on epidermal growth factor (EGF) pioneered the way to characterization of growth factor receptor interactions at the plasma membrane, ligand-dependent internalization and metabolism of receptors, and the discovery that growth factor receptors have ligand-activated tyrosine-specific kinase activity (194–197). Investigators turn to cell culture for purification and biochemical analysis of hormones and receptors, especially those of the human species. The human carcinoma cell line, A431, which produces a dramatic 3×10^6 molecules of EGF receptors per cell provided the abundant starting material for purification and analysis of this molecule (194–197). A human melanoma cell line provided the 10^6 molecules per cell sufficient to raise antibody to identify the human nerve growth factor receptor (198), and a human T cell line served a similar purpose for identification of the receptor for the T cell activator, interleukin-2 (199). Cells cultivated in roller bottles were used to isolate sufficient platelet-derived growth factor (PDGF) receptors for structural studies (200). Specific cell lines secreted insulin-like growth factor type two (IGF-2) (201–203) and the EGF homolog, transforming growth factor alpha (TGF-α) (166,167), in sufficient quantities for characterization.

Cultured cells are revealing the mechanism of synthesis and metabolism of polypeptide hormones. The structure of the mRNA for polypeptide regulators of cultured cell behavior suggests that most are translated as precursors that facilitate their secretion and access to external membrane receptors. Precursor polypeptides to factors such as epidermal growth factor (EGF) (204), transforming growth factor alpha (TGF-α) (205) and colony-stimulating factor one (CSF-1) (206) appear to be inserted and fixed into cell membranes prior to maturation of the factor by proteolysis. The membrane-bound precursor has a structure characteristic of transmembrane receptors and appears to be mitogenically active on other cells similar to mature factor (204,205). Transforming growth factor beta (TGF-β) appears to be processed and activated to mature form after secretion into the medium of cells (207–210). Heparin-binding (fibroblast) growth factors one and two (HBGF-1/HBGF-2) and interleukin-1 (211) are translated as mature polypeptides whose mechanism of appearance in the external environment of cells remains a question (105,106). Although they are secreted from tissue cells, platelet-derived factors such as PDGF and TGF-β are stored in the α-granules of platelets and delivered at the site of platelet attachment and aggregation (68,69,74,75).

Cultured cells will continue to be the means of identifying new activities, and the source for purification, characterization and cloning of new cellular regulators and their receptors. The proximal signal generated by polypeptide hormone receptors which have no apparent enzyme activity such as nerve growth factor (198,212), interleukin-1 (213), interleukin-6 (214), growth hormone (215) and prolactin (216), remains to be determined. Cell cultures for which the effectors of cell behavior have been identified will continue to present the experimental models to dissect out the cascade of events and genes that are activated by the hormone-receptor interaction (165,168,169, 194–197).

Metabolic pathways/enzymology. Mammalian cell lines bearing mutations in whole or specific steps of diverse metabolic pathways and advances in the culture of tissue-specific cell types in defined environments offer the biochemist sufficient material to discover and dissect cell type-specific metabolic pathways and their regulation. Cultured cells revealed the novel tyrosine kinase class of enzymes (52). They also revealed that some hormone receptors are allosterically-regulated enzymes, whose regulatory and catalytic domains are normally separated by the cell membrane, and whose loss of allosteric regulation causes unregulated cell growth (52, 160–163, 194–197).

A major challenge to the biochemist is to show which of the encyclopedic list of individual metabolic pathways and products that correlate with specific behavior of cultured cells actually causes the behavior.

Chemical carcinogenesis/toxicology/pharmacology. Mammalian cell cultures, especially in defined environments, provide the means to study the proximal effect and metabolism of carcinogens, toxins and drugs on specific cell types. The mechanism of drug resistance and gene amplification in vivo has been aided by studies of drug resistance and response to heavy metal ions in cultured cells (217,218).

Polycyclic aromatic hydrocarbons cause changes in properties of cultured cells that correlate with tumor cells in vivo (219). Major challenges remain to determine whether the molecular mechanisms underlying chemical alteration of isolated cell behavior can be unified with those emerging from viral and direct DNA-mediated transformation of cultured cells. Quantitative cell culture assays have been described which may rival and at least supplement the popular bacterial/microsomal Ames genotoxic test (220) for quantitative analysis and assessment of risk of environmental chemicals (221–224). Considerable progress has been made in validating mammalian cell cultures as supplements to animal exposure-response tests, such as the popular LD50/ED50 parameter using rodent populations and the Draize rabbit ocular irritation tests, for routine assessment of risk/benefit ratio of consumer drugs and environmental chemicals (221–224).

Plant diterpenes, especially 12-0-tetradecanoylphorbol-13-acetate (TPA), are potent tumor-promoting agents in vivo and mimic many changes in cultured cell behavior that are characteristic of cancer cells in vivo (226). In contrast to constitutive effects of viruses and chemicals, the TPA effect is reversible and usually induces a subset of the changes induced by viruses and chemicals. The identification of protein kinase C (PKC) as the receptor for TPA and the discovery that (i) some polypeptide growth factors activate PKC; (ii) growth factor receptors are targets of PKC activity; and (iii) a variety of ligand/receptor interactions on the cell surface results in production of diacylglycerol, the natural activator of PKC, revealed a common metabolic pathway by which tumor promoters and some growth factors may cause alterations in cultured cell behavior (225,227). The challenge remains to identify the substrates of PKC that cause alterations in cell behavior that is elicited by agents which activate the enzyme in cultured cells.

Hematopoiesis and immunology. The technique of clonal growth of hemopoietic progenitor cells in cultures of splenic and bone marrow cells was the key to sorting out hemopoietic cell lineages and the discovery of the polypeptide hormones known as hemopoietic colony-stimulating factors (CSF) (33,34) and lymphokines (35–38). Myeloma cell lines and hybridoma techniques, a result of cell fusion techniques, revolutionized immunology and many aspects of other biological sciences (50). The cloning of the immunoglobulin gene from myeloma cells led to new concepts in molecular biology and proved the somatic theory of generation of antibody diversity (51). Isolation and cloning of T cell antigen receptors, their mechanism of action and their relation to histocompatibility antigens was a result of the development of antigen-specific T cell lines and T cell hybridomas (51,228,229). Cultured cells will continue to play a major role in development of markers that define sublineages of hemopoietic cells and the cellular and molecular basis of specificity of the human response.

Developmental biology and embryology. Cell lines derived from embryonic carcinomas (teratomas) have provided stable and reproducible culture systems to study the process of differentiation and have yielded insight into the relationship between growth, malignancy and differentiation (230–234). Cultured embryonic stem cells bearing specific genetic mutations or artificially introduced genes can be mixed with early embryos (blastocysts) and upon implantation give rise to lines of transgenic animals carrying the mutations (235–237). Remarkable progress has occurred in maintenance and manipulation of germ cells and of pre-implantation embryos prior to implantation in animals and even humans. However, a major challenge remains to develop cell culture methods for step-by-step dissection of the factors that control development of pre-implantation and early post-implantation embryos.

Complex tissue physiology. Application of the general principles of defined cell culture environments allows dissection, prediction and reconstruction of the cellular and molecular events that unfold within complex tissues both in health and disease. Reservations about the utility and validity of the technique continue to diminish as application of the analytical methods and tools of protein chemistry, recombinant molecular biology and immunochemistry reveal which phenomena in cell culture reflects similar phenomena in tissues.

Vascular and liver tissue are leading prototypes of tissues whose component cell types in a defined environment are yielding considerable insight into contribution of different cell types to the events that may unfold in complex tissues upon transient or chronic perturbation. Blood vessel repair appears to occur in response to a network of polypeptide factors (77,238-242) which originate from both blood cells (platelets and monocytes) (68,69,74,75) and the endothelial cells (EC) and smooth muscle cells (SMC) which compose the tissue (238-245). Platelet-derived growth factor (PDGF) specifically promotes SMC growth with no effect on EC (77) which have no receptor for PDGF (246). Both EC (238-240) and SMC (243-245) synthesize and secrete additional PDGF into the microenvironment. Platelet-derived transforming growth factor beta (TGF-β) retards EC proliferation, but augments SMC proliferation (77). Both EC and SMC co-express both heparin-binding (fibroblast) growth factors type one and two (HBGF-1 and HBGF-2), but total HBGF expression is much higher in SMC and is predominately HBGF-1 (238-240,247). Monocyte-derived cytokines such as interleukin one (IL-1) and tumor necrosis factor (TNF) indirectly augment SMC proliferation by stimulation of PDGF synthesis and secretion (248) and up-regulation of the HBGF receptor in SMC, but dampen EC proliferation by down-regulation of the HBGF receptor in EC (249). Remodeling of the extracellular matrix by proteinases and other enzymes (238-242) affects release, activity and effective lifetime of the HBGF's in the microenvironment (105,106). Plasmin activity, most likely generated by HBGF activation of plasminogen activator in EC (106,241,242), selectively inactivates HBGF-1 (250) and may be the activator of latent platelet-derived TGF-β (208-210). Chronic activation of the network at certain blood vessel sites may explain the failure of EC to adequately regenerate and the SMC hyperplasia associated with atherosclerotic plaques (68,69,77,243-245).

The liver is composed of multiple cell types whose remarkable capacity for regeneration and expression of diverse genes are subject to manipulation, dissection and reconstitution both in vivo and in vitro (251-255). Careful study of the soluble and extracellular matrix factors produced by liver cells during regeneration in vivo into cultures of isolated hepatocytes are revealing that liver parenchymal cell proliferation appears to occur in linear steps from cells with large growth potential and reduced expression of liver-specific genes toward cells with less capacity for growth and higher levels of expression of liver-specific genes (64-66,76,78). The sequence appears to unfold by sequential expression of heparin-binding(acidic fibroblast) growth factor type one (HBGF-1), transforming growth factor alpha (TGF-α) and TGF-β. HBGF-1 is first activated in parenchymal cells which expands a proliferative population by an autocrine mechanism that is relatively resistant to the parenchymal cell growth inhibitor, TGF-β (78). The proliferative population is further expanded by expression of autocrine action of TGF-α (78,256). The magnitude of the response to TGF-α is limited by TGF-β by a paracrine mechanism (77,256). Activation of TGF-β lags both HBGF-1 and TGF-α after liver damage and occurs only in non-parenchymal cells (77,256). TGF-α action induces a low-affinity HBGF receptor phenotype in the parenchymal cells whose occupancy may dampen the mitogenic effect of HBGF (77) while TGF-β down-regulates the high-affinity mitogenic HBGF receptor (M. Kan, W. L. McKeehan, unpublished results). Specific elements of extracellular matrix produced by liver cells during the regeneration sequence modify responsiveness of cells to soluble polypeptide factors, modify the activity and lifetime of the heparin-binding (fibroblast) growth factors (64-66,76,77,105) in the local environment, and modify the quantitative level of expression of specific liver genes, apparently by effects on mRNA stability (72,73). Discrete liver cell populations and lineages can be defined and selected by expression of and responsiveness to specific growth factors and elements of the extracellular matrix.

Isolation of lineages of parenchymal cells from liver and other organs possessing large growth potential hold promise for correction of organ-specific diseases by insertional mutagenesis and transplantation of the cultured cells (257-259). Therapeutic application of cultured cells can involve seed stocks of cells with adequate growth potential to develop into an organoid in vivo (260) or construction of living tissue equivalents suitable for direct transplant (261-264). Living tissue equivalents that survive upon transplantation in animals have been constructed from isolated cells that compose human skin (261,262), thyroid (263) and blood vessels (264). Advances have been made in introducing foreign genes by insertional mutagenesis into the cultured skin and blood vessel cells from which the transplantable equivalents were constructed (265-267). Since survival and development of reconstructed tissues and organs is dependent on vascularization and blood supply, co-construction of specific organs from both the parenchymal cells and cells of the vascular system may be required (268). These advances suggest that the major goal of tissue culture research, e.g. isolation of specific cell types and their environment in vitro followed by reconstruction of them into tissues and organs, is coming to fruition.

Industrial Scale Mammalian Cell Products

Bioactive secretory proteins from mammalian cells are useful for research, diagnosis and therapy. These substances are often produced in trace amounts that preclude isolation of sufficient quantities in highly purified form from tissues and fluids especially human. Microbial recombinant technologies are the method of choice for production of these proteins. However, in many cases the prokaryote products are inactive, unstable or antigenic because of the absence of post-translational modification by mammalian processes.

Cell culture offers the means to produce these products and accumulate them in the extracellular medium in relatively pure form from normal differentiated cells or cells expressing specific genes introduced artificially (269-272). Several practical problems have begun to be

solved: (i) construction of defined media for maintaining differentiated functions of cells for protein production, (ii) efficient, cost-effective growth of engineered cell lines, (iii) enhancement of productivity of the bioactive proteins. However, unique problems associated with the maintenance of steady-state conditions, both in depletion of nutrients and generation of toxic products, in industrial scale cell cultures remain. For example, a plasma B cell in the lymph nodes secretes about 2000 antibody molecules per second, 1.7×10^8 molecules per day, or 43 picograms IgG per day. Under optimal conditions, the murine myeloma cell lines (MPC11) produce 5 picograms IgG/ cell/minute (20 to 30% of the total proteins synthesized), or about 3×10^{10} IgG molecules per day. Theoretically, cells in a 10 liter bioreactor at a density of 10^7 cells per ml could produce 720 grams of IgG per day. However, this enormous rate of synthesis cannot be maintained in current culture conditions and cells at a density of 10^6 per ml actually produce only about 1 gram IgG/10^{11} cells/day. It is expected that further advances in the basic biology of cultured cells and genetic engineering the host cell itself for optimum growth and secretory function will overcome these limitations.

PERSPECTIVE

Despite significant and gradual practical and fundamental contributions to eukaryotic biology over the past 70 years, the contributions of mammalian cell culture models and technologies have been modest in view of their enormous potential. No longer a cottage industry occupied with the phenomenon of cell growth and trial-and-error reconstruction of the complex extracellular milieu, mammalian cell culture has become a basic, if not essential, tool for fundamental discovery and elucidation of problems facing eukaryotic biologists. The current list of cellular regulators and their receptors likely represents a minority of those yet to be isolated and characterized. Post-receptor biochemical pathways that determine different parameters of cell behavior await elucidation.

The complete set of polypeptide regulators of growth and gene expression and completely defined environments have been determined for only a handful of the over 400 cell types in the body. Insertional mutagenesis has been achieved in only a few of these cell types. Living tissue equivalents from only a few of these cell types have reached clinical trials. Only a few mammalian cell products are produced on industrial scale and are in the marketplace. Despite the impressive list of genes that affect cell behavior and whose abnormal expression are associated with cancer and other pathologies, strategies for prevention and cure based on the discoveries are urgently needed. There is increasing public pressure to use cell cultures to reduce use of animals in assessment of risk to humans to environmental chemicals and pharmaceuticals. These opportunities are invitation for new investigators from various disciplines to take up the models and methodologies of mammalian cell culture.

REFERENCES

1. Schleiden, E. J. Beitrage zur phytogenesis. Arch. Anat. Physiol., Wiss. Med. (J. Muller), 1838:137-176; 1838.
2. Schwann, T. *Mikroskopische Untersuchungen uber die Ubereinstimmung in der Struktur und dem Wachsthum der Tiere und Pflanzen.* Ostwalds Klassiker der Exakten Wissenschaften. Berlin, Verlag der Sander schen Buchhandlung 176:1910; 1839.
3. Bernard, C. *Le, cons sur les phenomenes de la vie, communs aux animaux et aux vegetaux.* (2 Vols.), Paris, Bailliere; 1878-79.
4. Burrows, M. T. The cultivation of tissues of the chick embryo outside the body. J. Am. Med. Assoc. 55:2057-2058; 1910.
5. Carrel, A. On the permanent life of tissues outside of the organism. J. Exp. Med. 15:516-528; 1912.
6. Carrel, A. Tissue culture and cell physiology. Physiol. Rev. 4:1-20; 1924.
7. Gey, G. O.; Thalheimer, W. Observation of the effects of insulin introduced into the medium of tissue culture. J. Am. Med. Assoc. 82:1609; 1924.
8. Gey, G. O. An improved technique for massive culture. Am. J. Cancer 17:752-756; 1933.
9. Gey, G. O.; Gey, M. C. The maintenance of human normal cells and tumor cells in continuous culture. Am. J. Cancer 27:45-76; 1936.
10. Gey, G. O.; Seegar, G. E.; Hellman, L. M. The production of a gonadotropic substance (prolan) by placental cells in culture. Science 88:306-307; 1938.
11. Gey, G. O.; Coffman, W. D.; Kubicek, M. T. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res. 12:264-265; 1952.
12. Ehrmann, R. L.; Gey, G. O. The growth of cells on a transparent gel of reconstituted rat tail collagen. J. Natl. Cancer Inst. 16:1375-1415; 1956.
13. Earle, W. R. Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in living cells. J. Natl. Cancer Inst. 4:165-212; 1943.
14. Hayflick, L.; Moorhead, P. S. The serial cultivation of human diploid strains. Exp. Cell Res. 25:585-621; 1961.
15. Todaro, G. J.; Green, H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. 17:299-313; 1963.
16. Perry, R. P. The cellular sites of synthesis of ribosomal and 4S RNA. Proc. Natl. Acad. Sci. USA 48:2179-2186; 1962.
17. Scherrer, K.; Darnell, J. E. Sedimentation characteristics of rapidly labelled RNA from HeLa cells. Biochem. Biophys. Res. Commun. 7:486-490; 1962.
18. Penman, S.; Sherrer, K.; Becker, Y., et al. Polyribosomes in normal and poliovirus-infected HeLa cells and their relationship to messenger-RNA. Proc. Natl. Acad. Sci. USA 49:654-662; 1963.
19. Steiner, D. F.; Oyer, P. E. The biosynthesis of insulin and a probable precursor of insulin by a human islet adenoma. Proc. Natl. Acad. Sci. 57:473-480; 1967.
20. Steiner, D. F.; Clark, J. L.; Nolan, C., et al. Proinsulin and the biosynthesis of insulin. Recent Prog. Horm. Res. 25:207-282; 1969.
21. Reich, E.; Franklin, R. M.; Shatkin, A. J., et al. Effect of actinomycin D on cellular nucleic acid synthesis and virus production. Science 134:556-557; 1961.
22. Hsu, T. C. Mammalian chromosomes in vitro. J. Hered. 43:167-172; 1952.
23. Tjio, J. H.; Levan, A. The chromosome number of man. Separat ur Hereditas 42:1-6; 1956.
24. Huberman, J. A.; Riggs, A. D. On the mechanism of DNA replication in mammalian chromosomes. J. Mol. Biol. 32:327-341; 1968.
25. Caspersson, T.; Hulten, M.; Lindsten, J., et al. Identification of different Robertsonian translocations in man by quinacrine mustard fluorescence analysis. Hereditas 67:213-220; 1971.
26. Wang, H. C.; Fedoroff, S. Banding in human chromosomes treated with trypsin. Nature New Biol. 235:52-54; 1972.

27. Frye, L. D.; Edidin, M. The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. J. Cell. Sci. 7:319-335; 1970.
28. Temin, H. M.; Rubin, H. Characteristics of an assay for Rous Sarcoma virus and Rous Sarcoma cells in tissue culture. Virology 6:669-688; 1958.
29. Dulbecco, R. Viral carcinogenesis. Cancer Res. 21:975-980; 1961.
30. Stoker, M.; MacPherson, I. Studies on transformation of hamster cells by polyoma virus in vitro. Virology 14:359-370; 1961.
31. Sato, G. The role of serum in cell culture. In: Litwak, G., ed. *Biochemical Actions of Hormones*, Vol. 3, Academic Press, NY; 1975:391-396.
32. Barnes, D.; Sirbasku, D., eds., *Peptide Growth Factors*, Methods Enzymol., Vols. 146, 147, Academic Press, NY; 1987.
33. Metcalf, D.; Moore, M. A. S., eds., *Hematopoietic Cells*, North-Holland, Amsterdam (1971).
34. Clark, S. C.; Kamen, R. The human hematopoietic colony-stimulating factors. Science 236:1229-1237; 1987.
35. Pestka, S., ed. Interferons. *Methods Enzymol.* 79; 1981.
36. Sabato, G. D.; Langone, J. J.; Van Vunakis, H., eds. Immunochemical techniques. *Methods Enzymol.* 116H; 1985.
37. Clemens, M. J.; Morris, A. G.; Gearing, A. J. H., eds. *Lymphokines and Interferons*, IRL Press, Oxford; 1987.
38. Webb, D. R.; Pierce, C. W.; Cohen, S., eds. *Molecular Basis of Lymphokine Action*, Humana Press, NJ; 1988.
39. Hayashi, I.; Sato, G. Replacement of serum by hormones permits growth of cells in a defined medium. Nature 259:132-134; 1976.
40. Barnes, D.; Sato, G. Serum-free culture: a unifying approach. Cell 22:649-655; 1980.
41. Barnes, D. W.; McKeehan, W. L.; Sato, G. H. Cellular endocrinology: integrated physiology in vitro. In Vitro Cell. Devel. Biol. 23:659-662; 1987.
42. Bottenstein, J.; Hayashi, I.; Hutchings, S. The growth of cells in serum-free hormone-supplemented media. Methods Enzymol. 58:94-109; 1979.
43. Sato, G.; Ross, R., eds. *Hormones and Cell Culture*, Cold Spring Harbor, NY; 1979.
44. Sato, G. H.; Pardee, A. B.; Sirbasku, D. A., eds. *Growth of Cells in Hormonally Defined Media*, Cold Spring Harbor, NY; 1982.
45. Barnes, D. W.; Sirbasku, D. A.; Sato, G. H., eds. *Cell Culture Methods for Cell BIology*, Vols. 1-4, Alan R. Liss, NY; 1984.
46. Mather, J., ed. *Mammalian Cell Culture: The Use of Serum-Free Hormone-Supplemented Media*, Plenum Press, NY; 1984.
47. Bottenstein, J. E.; Sato, G., eds. *Cell Culture in the Neurosciences*, Vol. 1-3, Plenum Press, NY; 1985.
48. Taub, M., ed. *Tissue Culture of Epithelial Cells*, Plenum Press, NY; 1985.
49. Varmus, H. Retroviruses. Science 240:1427-1435; 1988.
50. Kohler, G.; Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495-497; 1975.
51. Tonegawa, S. Nobel Lecture in physiology or medicine-1987. Somatic generation of immune diversity. In Vitro Cell. Devel. Biol. 24:253-265; 1988.
52. Hunter, T.; Cooper, J. A. Protein-tyrosine kinases. Annu. Rev. Biochem. 54:897-930; 1985.
53. Bishop, J. M. Oncogenes. Sci. Am. 246:80-82; 1982.
54. Weinberg, R. A. The action of oncogenes in the cytoplasm and nucleus. Science 230:770-776; 1985.
55. Hunter, T. Oncogenes and proto-oncogenes: how do they differ? J. Natl. Cancer Inst. 73:773-786; 1984.
56. McKeehan, W. L.; Adams, P. S.; Rosser, M. P. Direct mitogenic effects of insulin, epidermal growth factor, glucocorticoid, cholera toxin, unknown pituitary factors and possibly prolactin, but not androgen, on normal rat prostate epithelial cells in serum-free, primary cell culture. Cancer Res. 44:1998-2010; 1984.
57. McKeehan, W. L.; Adams, P. S. Assay of growth factors for prostate epithelial cells. J. Tissue Culture Methods 10:151-154; 1986.
58. Crabb, J. W.; Armes, L. G.; Carr, S. A., et al. Complete primary structure of prostatropin, a prostate epithelial cell growth factor. Biochemistry 25:4988-4993; 1986.
59. McKeehan, W. L.; Adams, P. S.; Fast, D. Different hormonal requirements for androgen-independent growth of normal and tumor epithelial cells from rat prostate. In Vitro Cell. Devel. Biol. 23:147-152; 1987.
60. Ham, R. G.; McKeehan, W. L. Media and growth requirements. *Methods Enzymol.* 58:44-93; 1979.
61. Ham, R. G. Survival and growth requirements of non-transformed cells. Handbook Exptl. Pharmacol. 57:13-88; 1981.
62. McKeehan, W. L.; McKeehan, K. A. Extracellular regulation of fibroblast multiplication: a direct kinetic approach to analysis of role of low molecular weight nutrients and serum growth factors. J. Supramol. Struct. Cell. Biochem. 15:83-110; 1981.
63. Bettgar, W. J.; Ham, R. G. The nutrient requirements of cultured mammalian cells. Adv. Nutr. Res. 4:249-286; 1982.
64. Reid, L.; Jefferson, D. Cell culture studies using extracts of extracellular matrix to study growth and differentiation in mammalian cells. In: Mather, J., ed. *Mammalian Cell Culture*, Plenum Press, NY; 1984:239-280.
65. Reid, L. M.; Abren, S. L.; Montgomery, K. Extracellular matrix and hormonal regulation of synthesis and abundance of messenger RNAs in cultured liver cells. In: Arias, I. M.; Jakoby, W. B.; Popper, H., et al., eds. *The Liver: Biology and Pathobiology*, Raven Press; 1988:717-737.
66. Reid, L. M. Generic methods for defined hormonal and matrix conditions for studies of growth of gene expression in differentiated epithelia. In: Pollard, J. W.; Walker, J. M., eds. *Methods in Molecular Biology*, Vol. 5, Humana Press, Inc., in press, 1989.
67. Sporn, M. B.; Todaro, G. J. Autocrine secretion and malignant transformation of cells. N. Engl. J. Med. 303:878-880; 1980.
68. Ross, R.; Raines, E. W.; Bowen-Pope, D. F. The biology of platelet-derived growth factor. Cell 46:155-169; 1986.
69. Deuel, T. F. Polypeptide growth factors: roles in normal and abnormal cell growth. Annu. Rev. Cell Biol. 3:443-492; 1987.
70. Stampfer, M. R. Cholera toxin stimulation of human mammary epithelial cells in culture. In Vitro 18:531-537; 1982.
71. Rheinwald, J. G.; Green, H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331-343; 1975.
72. Clayton, D. F.; Darnell, J. E., Jr. Changes in liver-specific compared to common gene transcription during primary culture of mouse hepatocytes. Mol. Cell. Biol. 3:1552-1561; 1983.
73. Jefferson, D. M.; Clayton, D. F.; Darnell, J. E., Jr., et al. Posttranscriptional modulation of gene expression in cultured rat hepatocytes. Mol. Cell. Biol. 4:1929-1934; 1984.
74. Sporn, M. B.; Roberts, A. B.; Wakefield, L. M., et al. Transforming growth factor-beta: biological function and chemical structure. Science 233:532-534; 1986.
75. Roberts, A. B.; Sporn, M. B. Transforming growth factor beta. Adv. Cancer Res. 51:107-145; 1988.
76. Kan, M.; DiSorbo, D.; Hou, J., et al. High and low affinity binding of heparin-binding growth factor to a 130-kDa receptor correlates with stimulation and inhibition of growth of a differentiated human hepatoma cell. J. Biol. Chem. 263:11306-11313; 1988.
77. Hoshi, H.; Kan, M.; Chen, J. K., et al. Comparative endocrinology-paracrinology-autocrinology of human adult large vessel endothelial and smooth muscle cells. In Vitro Cell. Devel. Biol. 24:309-320; 1988.
78. Kan, M.; Huang, J.; Mansson, P.-E., et al. Heparin-binding growth factor type one (acidic fibroblast growth factor): a potential biphasic autocrine and paracrine regulator of hepatocyte regeneration. Proc. Natl. Acad. Sci. USA 86:7432-7436; 1989.
79. Puck, T. T., ed. *The Mammalian Cell as a Microorganism*, Holden-Day, Inc., San Francisco; 1975.

80. Fischer, A. Die bedeutung der aminosauren fur die gewelezellen in vitro. Acta. Physiol. Scand. 2:145-148; 1941.
81. White, P. R. Cultivation of animal tissues in vitro in nutrients of precisely known constitution. Growth 10:231-239; 1946.
82. Morgan, J. F.; Morton, H. J.; Parker, R. C. Nutrition of animal cells in tissue culture. Proc. Soc. Exp. Biol. Med. 73:1-8; 1950.
83. Morton, H. J.; Pasieka, A. E.; Morgan, J. F. The nutrition of animal cells cultivated in vitro. J. Biophys. Biochem. Cytol. 2:589-596; 1956.
84. Waymouth, C. A serum-free nutrient solution sustaining rapid and continuous proliferation of strain L (Earle) mouse cells. J. Natl. Cancer Inst. 17:315-325; 1956.
85. Evans, V. J.; Bryant, J. C.; McQuilkin, W. T., et al. Studies of nutrient media for tissue cells in vitro. Cancer Res. 16:87-94; 1956.
86. Moore, G. E.; Gerner, R. E.; Franklin, H. A. Culture of normal human leukocytes. J. Am. Med. Assoc. 199:519-524; 1967.
87. Eagle, H. Nutrition needs of mammalian cells in tissue culture. Science 122:501-504; 1955.
88. Eagle, H.; Piez, K. The population-dependent requirement by cultured mammalian cells for metabolites which they can synthesize. J. Exp. Med. 116:29-43; 1962.
89. McKeehan, W. L.; Hamilton, W. G.; Ham, R. G. Selenium is an essential nutrient for growth of WI-38 diploid human fibroblasts. Proc. Natl. Acad. Sci. USA 73:2023-2027; 1976.
90. Kano-Sueoka, T.; Cohen, D. M.; Yamaizumi, Z., et al. Phosphoethanolamine as a growth factor of a mammary carcinoma cell line of rat. Proc. Natl. Acad. Sci. USA 76:5741-5744; 1979.
91. Bettger, W. J.; Boyce, S. T.; Walthall, B. J., et al. Rapid clonal growth and serial passage of human diploid fibroblasts in a lipid-enriched synthetic medium supplemented with epidermal growth factor, insulin, and dexamethasone. Proc. Natl. Acad. Sci. USA 78:5588-5592; 1981.
92. Tsao, M. C.; Walthall, B. J.; Ham, R. G. Clonal growth of normal human epidermal keratinocytes in a defined medium. J. Cell. Physiol. 110:219-229; 1982.
93. Hammond, S. L.; Ham, R. G.; Stampfer, M. R. Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc. Natl. Acad. Sci. USA 81:5435-5439; 1984.
94. Bettger, W. J.; McKeehan, W. L. Mechanisms of cellular nutrition. Physiol. Rev. 66:1-35; 1986.
95. McKeehan, W. L.; McKeehan, K. A. Epidermal growth factor modulates extracellular Ca^{2+} requirement for multiplication of normal human skin fibroblasts. Exp. Cell Res. 123:397-400; 1979.
96. McKeehan, W. L.; McKeehan, K. A. Serum factors modulate the cellular requirements of Ca^{2+}, K^+, Mg^{2+}, phosphate ions, and 2-oxocarboxylic acids for multiplication of normal human fibroblasts. Proc. Natl. Acad. Sci. USA 77:3417-3421; 1980.
97. Newsholme, E. A.; Crabtree, B.; Ardawi, M. S. The role of high rates of glycolysis and glutamine utilization in rapidly dividing cells. Biosci. Rep. 5:393-400; 1985.
98. McKeehan, W. L. Glutaminolysis in animal cells. In: Morgan, M. J., ed. *Carbohydrate Metabolism in Cultured Cells*, Plenum, London, 1986:111-150.
99. McKeehan, W. L.; McKeehan, K. A.; Calkins, D. Extracellular regulation of fibroblast multiplication. Quantitative differences in nutrient and serum factor requirements for multiplication of normal and SV40-virus transformed human lung cells. J. Biol. Chem. 256:2973-2981; 1981.
100. Salmon, W.; Daughaday, W. A hormonally-controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. J. Lab. Clin. Med. 49:825-836; 1957.
101. Clemmons, D. R.; Van Wyk, J. J. Somatomedin: physiological control and effects on cell proliferation. In: Baserga, R., ed. *Tissue Growth Factors, Handbook of Experimental Pharmacology*. Springer-Verlag, New York, Vol. 57, 1981:161-208.
102. Froesch, E. R.; Schmid, C.; Schwander, J., et al. Actions of insulin-like growth factors. Annu. Rev. Physiol. 47:443-467; 1985.
103. Sirbasku, D. A. Estrogen induction of growth factors specific for hormone-responsive mammary, pituitary, and kidney tumor cells. Proc. Natl. Acad. Sci. USA 75:3786-3790; 1978.
104. James, R.; Bradshaw, R. A. Polypeptide growth factors. Annu. Rev. Biochem. 53:259-292; 1984.
105. Burgess, W. H.; Maciag, T. The heparin-binding (fibroblast) growth factor family of proteins. Annu. Rev. Biochem. 58:575-606; 1989.
106. Rifkin, D. B.; Moscatelli, D. Recent developments in the cell biology of basic fibroblast growth factor. J. Cell Biol. 109:1-6; 1989.
107. Rosenberg, R. D.; Lam, L. Correlation between structure and function of heparin. Proc. Natl. Acad. Sci. USA 76:1218-1222; 1979.
108. Fedarko, N. S.; Conrad, H. E. A unique heparan sulfate in the nuclei of hepatocytes: structural changes with the growth state of the cells. J. Cell Biol. 102:587-599; 1986.
109. Li, M. L.; Aggeler, J.; Farson, D. A., et al. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. Proc. Natl. Acad. Sci. USA 84:136-140; 1987.
110. Vlodavsky, I.; Folkman, J.; Sullivan, R., et al. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. Proc. Natl. Acad. Sci. USA 84:2292-2296; 1987.
111. Spray, D. C.; Fujita, M.; Saez, J. C., et al. Proteoglycans and glycosaminoglycans induce gap junction synthesis and function in primary liver cultures. J. Cell Biol. 105:541-551; 1987.
112. Rojkind, M.; Gatmaitan, Z.; Mackensen, S., et al. Connective tissue biomatrix: its isolation and utilization for long-term cultures of normal rat hepatocytes. J. Cell. Biol. 87:255-263; 1980.
113. Muschel, R.; Khoury, G.; Reid, L. M. Regulation of insulin mRNA abundance and adenylation: dependence on hormones and matrix substrata. Mol. Cell. Biol. 6:337-341; 1986.
114. Kleinman, H. K.; Luckenbill-Edds, L.; Cannon, F. W., et al. Use of extracellular matrix components for cell culture. Anal. Biochem. 166:1-13; 1987.
115. Ailenberg, M.; Tung, P. S.; Pelletier, M., et al. Modulation of Sertoli cell functions in the two-chamber assembly by peritubular cells and extracellular matrix. Endocrinology 122:2604-2612; 1988.
116. Hynes, R. O. Integrins: a family of cell surface receptors. Cell 48:549-554; 1987.
117. Ruoslahti, E.; Pierschbacher, M. D. New perspectives in cell adhesion: RGD and integrins. Science 238:491-497; 1987.
118. Ignotz, R. A.; Endo, T.; Massague, J. Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-beta. J. Biol. Chem. 262:6443-6446; 1987.
119. Chen, J. K.; Hoshi, H.; McKeehan, W. L. Transforming growth factor type beta specifically stimulates synthesis of proteoglycan in human adult arterial smooth muscle cells. Proc. Natl. Acad. Sci. USA 84:5287-5291; 1987.
120. Ignotz, R. A.; Massague, J. Cell adhesion protein receptors as targets for transforming growth factor-beta action. Cell 51:189-197; 1987.
121. Rizzino, A. Transforming growth factor-beta: multiple effects on cell differentiation and extracellular matrices. Devel. Biol. 130:411-422; 1988.
122. Puck, T. T.; Marcus, P. I. A rapid method for viable cell titration and clone production with HeLa cells in tissue culture. Proc. Natl. Acad. Sci. USA 41:432-437; 1955.
123. Puck, T. T.; Marcus, P. I.; Cieciura, S. J. Clonal growth of mammalian cells in vitro. J. Exp. Med. 103:273-283; 1956.
124. Ham, R. G.; Puck, T. T. Quantitative colonial growth of isolated mammalian cells. Methods Enzymol. 5:90-119; 1962.
125. Kao, F. T.; Puck, T. T. Genetics of somatic mammalian cells, VII. Induction and isolation of nutritional mutants in Chinese hamster cells. Proc. Natl. Acad. Sci. USA 60:1275-1281; 1968.
126. Thompson, L. H.; Mankovitz, R.; Baker, R. M., et al. Isolation of temperature-sensitive mutants of L-cells. Proc. Natl. Acad. Sci. USA 66:377-384; 1970.
127. Choi, K. W.; Bloom, A. D. Cloning human lymphocytes in vitro. Nature 227:171-173; 1970.

128. Konigsberg, I. R. Clonal analysis of myogenesis. Science 140:1273-1284; 1963.
129. Coon, H. G. Clonal stability and phenotypic expression of chick cartilage cells in vitro. Proc. Natl. Acad. Sci. USA 55:66-73; 1966.
130. Coon, H. G.; Cahn, R. D. Differentiation in vitro: effects of Sephadex fractions of chick embryo extract. Science 153:1116-1119; 1966.
131. Cahn, R. D.; Cahn, M. B. Heritability of cellular differentiation: clonal growth and expression of differentiation in retinal pigment cells in vitro. Proc. Natl. Acad. Sci. USA 55:106-114; 1966.
132. Ham, R. G. Cloning of mammalian cells. Met. Cell. Physiol. 5:37-74; 1972.
133. Sanford, K. K.; Earle, W. R.; Likely, G. D. The growth in vitro of single isolated cells. J. Natl. Cancer Inst. 9:229-246; 1948.
134. Harrison, R. G. Observations on the living developing nerve fiber. Proc. Soc. Exp. Biol. Med. 4:140-143; 1907.
135. Harrison, R. G. The outgrowth of the nerve fiber as a mode of protoplasmic movement. J. Exp. Zool. 9:787-848; 1910.
136. Sato, G.; Zaroff, L.; Mills, S. E. Tissue culture populations and their relation to the tissue of origin. Proc. Natl. Acad. Sci. USA 46:963-972; 1960.
137. Schindler, R.; Day, M.; Fischer, G. A. Culture of neoplastic mast cells and their synthesis of 5-hydroxy-tryptamine and histamine in vitro. Cancer Res. 19:47-51; 1959.
138. Thompson, E. B.; Tomkins, G. M.; Curran, J. F. Induction of tyrosine alpha-ketoglutarate transaminase by steroid hormones in a newly established tissue culture cell line. Proc. Natl. Acad. Sci. USA 56:296-303; 1966.
139. Yaffe, D. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc. Natl. Acad. Sci. USA 61:477-483; 1968.
140. Leighton, J.; Estes, L. W.; Mansukhani, S., et al. A cell line derived from normal dog kidney (MDCK) exhibiting qualities of papillary adenocarcinoma and of renal tubular epithelium. Cancer 26:1022-1028; 1970.
141. Knowles, B. B.; Howe, C. C.; Aden, D. P. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 209:497-499; 1980.
142. Buonassisi, V.; Sato, G.; Cohen, A. I. Hormone-producing cultures of adrenal and pituitary tumor origin. Proc. Natl. Acad. Sci. USA 48:1184-1190; 1962.
143. Yasamura, Y.; Tashjian, A. H., Jr.; Sato, G. H. Establishment of four functional, clonal strains of animal cells in culture. Science 154:1186-1189; 1966.
144. Mohit, B.; Sato, G. H. Improved in vitro survival of normal, functional spleen cells. Science 157:449-451; 1967.
145. Augusti-Tocco, G.; Sato, G. Establishment of functional clonal lines of neurons from mouse neuroblastoma. Proc. Natl. Acad. Sci. USA 64:311-315; 1969.
146. Sato, G.; Augusti-Tocco, G.; Posner, M. Hormone-secreting and hormone-responsive cell cultures. Recent Prog. Horm. Res. 26:539-546; 1970.
147. Rosenthal, M. D.; Wishnow, R. M.; Sato, G. H. In vitro growth and differentiation of clonal populations of multipotential mouse cells derived from a transplantable testicular teratocarcinoma. J. Natl. Cancer Inst. 44:1001-1014; 1970.
148. Sato, G., ed. *Functionally Differentiated Cell Lines*, Alan R. Liss, NY; 1981.
149. Hammond, S. L.; Ham, R. G.; Stampfer, M. R. Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc. Natl. Acad. Sci. USA 81:5435-5439; 1984.
150. Sanford, K. K.; Likely, G. D.; Earle, W. R. The development of variations in transplantability and morphology within a clone of mouse fibroblasts transformed to sarcoma-producing cells in vitro. J. Natl. Cancer Inst. 15:215-230; 1954.
151. Abercrombie, M.; Heaysman, J. E. M. Observations on the social behavior of cells in tissue culture. Exp. Cell Res. 6:293-306; 1954.
152. Stoker, M.; MacPherson, I. Studies on transformation of hamster cells by polyoma virus in vitro. Virology 14:359-370; 1961.
153. Todaro, G. J.; Green, H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. 17:299-313; 1963.
154. MacPherson, I.; Montagnier, L. Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virology 23:291-294; 1964.
155. Holley, R. W.; Kiernan, J. A. "Contact inhibition" of cell division in 3T3 cells. Proc. Natl. Acad. Sci. USA 60:300-304; 1964.
156. Aaronson, S. A.; Todaro, G. J. Basis for the acquisition of malignant potential by mouse cells cultivated in vitro. Science 162:1024-1026; 1968.
157. Pollack, R. E.; Green, H.; Todaro, G. J. Growth control in cultured cells: selection of sublines with increased sensitivity to contact inhibition and decreased tumor-producing ability. Proc. Natl. Acad. Sci. USA 60:126-133; 1968.
158. Dulbecco, R. Topoinhibition and serum requirement of transformed and untransformed cells. Nature 227:802-806; 1970.
159. Freedman, V. H.; Shin, S. I. Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. Cell 3:355-359; 1974.
160. Doolittle, R. F.; Hunkapiller, M. W.; Hood, L. E., et al. Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221:275-277; 1983.
161. Waterfield, M. D.; Scrace, G. T.; Whittle, N., et al. Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. Nature 304:35-39; 1983.
162. Downward, J.; Yarden, Y.; Mayes, E., et al. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature 307:521-527; 1984.
163. Sherr, C. J.; Rettenmier, C. W.; Sacca, R., et al. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41:665-676; 1985.
164. Stiles, C. D. The molecular biology of platelet-derived growth factor. Cell 33:653-655; 1983.
165. Rollins, B. J.; Stiles, C. D. Regulation of c-myc and c-fos proto-oncogene expression by animal cell growth factors. In Vitro Cell. Devel. Biol. 24:81-84; 1988.
166. De Larco, J. E.; Todaro, G. J. Growth factors from murine sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. USA 75:4001-4005; 1978.
167. Derynck, R. Transforming growth factor-alpha: structure and biological activities. J. Cell. Biochem. 32:293-304; 1986.
168. Cochran, B. H.; Zumstein, P.; Zullo, J., et al. Differentiation colony hybridization: molecular cloning from a zero data base. Methods Enzymol. 147:64-85; 1987.
169. Scher, C. D.; Pledger, W. J. Identification of platelet-derived growth factor-modulated proteins. Methods Enzymol. 147:85-92; 1987.
170. Okada, Y. The fusion of Ehrlich's tumor cells caused by HVJ virus in vitro. Biken J. 1:103-110; 1958.
171. Harris, H. Behaviour of differentiated nuclei in heterokaryons of animal cells from different species. Nature 206:583-588; 1965.
172. Pontecorvo, G. Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. Somatic Cell Genet. 1:397-400; 1975.
173. Ruddle, F. H. A new era in mammalian gene mapping: somatic cell genetics and recombinant DNA methodologies. Nature 294:115-120; 1981.
174. Shows, T. B.; Sakaguchi, A. Y.; Naylor, S. L. Mapping the human genome, cloned genes, DNA polymorphisms, and inherited disease. Adv. Hum. Genet. 12:341-452; 1982.
175. Ege, T.; Ringertz, N. R. Viability of cells reconstituted by virus-induced fusion of minicells with anucleate cells. Exp. Cell Res. 94:469-473; 1975.
176. Fournier, R. E.; Ruddle, F. H. Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells. Proc. Natl. Acad. Sci. USA 74:319-323; 1977.

177. Tunnacliffe, A.; Goodfellow, P. Attached cell antigen 28.3.7 mapping to human chromosome 15 characterizes TPA-induced differentiation of the promyelocytic HL-60 cell line to give macrophage/monocyte populations. EMBO J. 2:2007-2012; 1983.
178. Saxon, P. J.; Srivatsan, E. S.; Leipzig, G. V., et al. Selective transfer of individual human chromosomes to recipient cells. Mol. Cell. Biol. 5:140-146; 1985.
179. Sager, R. Genetic suppression of tumor formation. Adv. Cancer Res. 44:43-68; 1985.
180. Harris, H. The genetic analysis of malignancy. J. Cell Sci. Suppl. 4:431-444; 1986.
181. Stanbridge, E. J. Genetic regulation of tumorigenic expression in somatic cell hybrids, Klein, G., ed. Advances in Viral Oncology. Vol. 6, Raven Press, New York; 1987:83-102.
182. Stanbridge, E. J. Suppression of malignancy in human cells. Nature 260:17-20; 1976.
183. Klinger, H. P. Suppression of tumorigenicity. Cytogenet. Cell Genet. 32:68-84; 1982.
184. Stanbridge, E. J. A case for human tumor-suppressor genes. Bioassays 3:252-255; 1985.
185. Klein, G. The approaching era of the tumor suppressor genes. Science 238:1539-1545; 1987.
186. Weissman, B. E.; Saxon, P. J. Pasquale, S. R., et al. Introduction of a normal human chromosome 11 into a Wilms' tumor cell line controls its tumorigenic expression. Science 236:175-180; 1987.
187. Stanbridge, E. J. A genetic basis for tumour suppression. In: Brock, G.; Marsh, J., eds. CIBA Found. Symp. No. 142, John Wiley and Sons, New York, 1989:149-159.
188. Davidson, R. L., ed. *Somatic Cell Hybridization*, Raven Press, New York; 1974.
189 Weiss, M. C.; Sparkes, R. S.; Bertolotti, R. Expression of differentiated functions in hepatoma cell hybrids: IX extinction and reexpression of liver-specific enzymes in rat hepatoma-Chinese hamster fibroblast hybrids. Somatic Cell Genet. 1:27-40; 1975.
190. Chin, A. C.; Fournier, R. E. A genetic analysis of extinction: trans-regulation of 16 liver-specific genes in hepatoma-fibroblast hybrid cells. Proc. Natl. Acad. Sci. USA 84:1614-1618; 1987.
191. Blau, H. M.; Chiu, C. P.; Webster, C. Cytoplasmic activation of human nuclear genes in stable heterocaryons. Cell 32:1171-1180; 1983.
192. Hardeman, E. C.; Chiu, C. P.; Minty, A., et al. The pattern of actin expression in human fibroblast x mouse muscle heterokaryons suggests that human muscle regulatory factors are produced. Cell 47:123-130; 1986.
193. Chin, A. C.; Fournier, R. E. Tissue-specific expression of hepatic functions. Genetics aspects. Ann. N.Y. Acad. Sci. 478:120-130; 1986.
194. Stoscheck, C. M.; Carpenter, G. Biology of the A-431 cell: a useful organism for hormone research. J. Cell. Biochem. 23:191-202; 1983.
195. Cohen, S. Epidermal growth factor. Nobel Lecture in Physiology or Medicine-1986. In Vitro Cell. Devel. Biol. 23:239-246; 1987.
196. Carpenter, G. Receptors for epidermal growth factor and other polypeptide mitogens. Annu. Rev. Biochem. 56:881-914; 1987.
197. Yarden, Y.; Ullrich, A. Molecular analysis of signal transduction by growth factors. Biochemistry 27:3113-3119; 1988.
198. Chao, M. V.; Bothwell, M. A.; Ross, A. H., et al. Gene transfer and molecular cloning of the human NGF receptor. Science 232:518-521; 1986.
199. Robb, R. J.; Greene, W. C.; Rusk, C. M. Low and high affinity cellular receptors for interleukin 2. J. Exp. Med. 160:1126-1146; 1984.
200. Yarden, Y.; Escobedo, J. A.; Kuang, W. J., et al. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature 323:226-232; 1986.
201. Dulak, N. C.; Temin, H. M. Multiplication-stimulating activity for chicken embryo fibroblasts from rat liver cell conditioned medium: a family of small polypeptides. J. Cell Physiol. 81:161-170; 1973.
202. Dulak, N. C.; Temin, H. M. A partially purified polypeptide fraction from rat liver cell conditioned medium with multiplication-stimulating activity for embryo fibroblasts. J. Cell Physiol. 81:153-160; 1973.
203. Greenstein, L. A.; Gaynes, L. A.; Romanus, J. A., et al. Purification of rat insulin-like growth factor II. Methods Enzymol. 146:259-269; 1987.
204. Mroczkowski, B.; Reich, M.; Chen, K., et al. Recombinant human epidermal growth factor precursor is a glycosylated membrane protein with biological activity. Mol. Cell. Biol. 9:2771-2778; 1989.
205. Wong, S. T.; Winchell, L. F.; McCune, B. K., et al. The TGF-alpha precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. Cell 56:495-506; 1989.
206. Kawasaki, E. S.; Ladner, M. B.; Wang, A. M., et al. Molecular cloning of a complementary DNA encoding human macrophage-specific colony-stimulating factor (CSF-1). Science 230:291-296; 1985.
207. Derynck, R.; Jarrett, J. A.; Chen, E. Y., et al. Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. Nature 316:701-705; 1985.
208. Lawrence, D. A.; Pircher, R.; Jullien, P. Conversion of a high molecular weight latent beta-TGF from chicken embryo fibroblasts into a low molecular weight active beta-TGF under acidic conditions. Biochem. Biophys. Res. Commun. 133:1026-1034; 1985.
209. Orlidge, A.; D'Amore, P. A. Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. J. Cell Biol. 105:1455-1462; 1987.
210. Lyons, R. M.; Keski-Oja, J.; Moses, H. L. Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. J. Cell Biol. 106:1659-1665; 1988.
211. March, C. J.; Mosley, B.; Larsen, A., et al. Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature 315:641-647; 1985.
212. Radeke, M. J.; Misko, T. P.; Hsu, C., et al. Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325:593-597; 1987.
213. Sims, J. E.; March, C. J.; Cosman, D., et al. cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. Science 241:585-589; 1988.
214. Yamasaki, K.; Taga, T.; Hirata, Y., et al. Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. Science 241:825-828; 1988.
215. Leung, D. W.; Spencer, S. A.; Cachianes, G., et al. Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature 330:537-543; 1987.
216. Boutin, J. M.; Jolicoeur, C.; Okamura, H., et al. Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. Cell 53:69-77; 1988.
217. Schimke, R. T. Gene amplification in cultured animal cells. Cell 37:705-713; 1984.
218. Gottesman, M. M.; Pastan, I. The multidrug transporter, a double-edged sword. J. Biol. Chem. 263:12163-12166; 1988.
219. Mishra, N.; Dunkel, M.; Mehlman, eds. *Mammalian Cell Transformation by Chemical Carcinogens*. Senate Press, Inc., Princeton Junction, NJ; 1980.
220. Ames, B. N.; Durston, W. E.; Yamasaki, E., et al. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc. Natl. Acad. Sci. USA 70:2281-2285; 1973.
221. International Conference on Practical In Vitro Toxicology, Food and Chemical Toxicology 24(6-7):447-818; 1986.
222. Tennant, R. W.; Margolin, B. H.; Shelby, M. D., et al. Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays. Science 236:933-941; 1987.
223. Balls, M. 4th International Workshop on In Vitro Toxicology. Crieff, Perthshire, Scotland, September 8-12, 1986. Xenobiotica 18(6):615-616; 1988.

224. Goldberg, A. M.; Frazier, J. M. Alternatives to animals in toxicity testing. Scientific American 261:24-30; 1989.
225. Nishizuka, Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661-665; 1988.
226. Weinstein, I. B. Effects of phorbol esters in cell culture and their relevance to short-term assays for tumor promoters. In: Mishra, N.; Dunkel, V.; Mehlman, M., eds. *Mammalian Cell Transformation by Chemical Carcinogens*, Vol. 1, Senate Press, Princeton Junction, NJ; 1980:427-446.
227. Michell, R. H.; Drummond, A. H.; Downes, C. P., eds. *Inositol Lipids in Cell Signalling*. Academic Press, NY; 1989.
228. Hedrick, S. M.; Cohen, D. I.; Nielsen, E. A., et al. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. Nature 308:149-153; 1984.
229. Yanagi, Y.; Yoshikai, Y.; Leggett, K., et al. A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. Nature 308:145-149; 1984.
230. Stevens, L. C. Experimental production of testicular teratomas in mice. Proc. Natl. Acad. Sci. USA 52:654-661; 1964.
231. Pierce, G. B. Teratocarcinoma: model for a developmental concept of cancer. Curr. Top. Dev. Biol. 2:223-246; 1967.
232. Nicolas, J. F.; Jakob, H.; Jacob, F. Teratocarcinoma-derived cell lines and their use in the study of differentiation. In: Sato, G., ed. *Functionally Differentiated Cell Lines*, Alan R. Liss, NY; 1981:185-210.
233. Evans, M. J.; Kaufman, M. H. Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154-156; 1981.
234. Martin, G. R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA 78:7634-7638; 1981.
235. Brinster, R. L. The effect of cells transferred into the mouse blastocyst on subsequent development. J. Exp. Med. 140:1049-1056; 1974.
236. Palmiter, R. D.; Brinster, R. L.; Hammer, R. E., et al. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300:611-615; 1982.
237. Mansour, S. L.; Thomas, K. R.; Capecchi, M. R. Disruption of the proto-oncogene int-2 mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature 336:348-352; 1988.
238. Weinstein, R.; Stemerman, M. B.; Maciag, T. Hormonal requirements for growth of arterial smooth muscle cells in vitro: an endocrine approach to atherosclerosis. Science 212:818-820; 1981.
239. Maciag, T.; Kadish, J.; Wilkins, L., et al. Organizational behavior of human umbilical vein endothelial cells. J. Cell. Biol. 94:511-520; 1982.
240. Winkles, J. A.; Friesel, R.; Burgess, W. H., et al. Human vascular smooth muscle cells both express and respond to heparin-binding growth factor I (endothelial cell growth factor). Proc. Natl. Acad. Sci. USA 84:7124-7128; 1987.
241. Moscatelli, D.; Rifkin, D. B. Membrane and matrix localization of proteinases: a common theme in tumor cell invasion and angiogenesis. Biochim. Biophys. Acta 948:67-85; 1988.
242. Saksela, O.; Rifkin, D. B. Cell-associated plasminogen activation: regulation physiological functions. Annu. Rev. Cell. Biol. 4:93-126; 1988.
243. Walker, L. N.; Bowen-Pope, D. F.; Ross, R., et al. Production of platelet-derived growth factor-like molecules by cultured arterial smooth muscle cells accompanies proliferation after arterial injury. Proc. Natl. Acad. Sci. USA 83:7311-7315; 1986.
244. Sejersen, T.; Betsholtz, C.; Sjolund, M., et al. Rat skeletal myoblasts and arterial smooth muscle cells express the gene for the A chain but not the gene for the B chain (c-sis) of platelet-derived growth factor (PDGF) and produce a PDGF-like protein. Proc. Natl. Acad. Sci. USA 83:6844-6848; 1986.
245. Barrett, T. B.; Benditt, E. P. Sis (platelet-derived growth factor B chain) gene transcript levels are elevated in human atherosclerotic lesions compared to normal artery. Proc. Natl. Acad. Sci. USA 84:1099-1103; 1987.
246. Heldin, C. H.; Westermark, B.; Wasteson, A. Specific receptors for platelet-derived growth factor on cells derived from connective tissue and glia. Proc. Natl. Acad. Sci. USA 78:3664-3668; 1981.
247. Mansson, P. E.; Malark, M.; Sawada, H., et al. Heparin-binding (fibroblast) growth factors type one and two genes are co-expressed in proliferating normal human vascular endothelial and smooth muscle cells. In Vitro Cell. Devel. Biol., in press; 1990.
248. Raines, E. W.; Dower, S. K.; Ross, R. Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA. Science 243:393-396; 1989.
249. Sawada, H.; Kan, M.; McKeehan, W. L. Differential modulation of proliferation and heparin-binding (fibroblast) growth factor binding to human aortic endothelial cells and smooth muscle cells by interleukin-1 and tumor necrosis factor. In Vitro Cell. Devel. Biol., in press; 1990.
250. Lobb, R. R. Thrombin inactivates acidic fibroblast growth factor but not basic fibroblast growth factor. Biochemistry 27:2572-2578; 1988.
251. Grisham, J. W. A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H^3. Cancer Res. 22:842-849; 1962.
252. Bucher, N. L. R.; Malt, R. A., eds. *Regeneration of Liver and Kidney*, Little Brown, Boston; 1971.
253. Friedman, J. M.; Chung, E. Y.; Darnell, J. E., Jr. Gene expression during liver regeneration. J. Mol. Biol. 179:37-53; 1984.
254. Baumann, H. Hepatic acute phase reaction in vivo and in vitro. In Vitro Cell. Devel. Biol. 25:115-126; 1989.
255. Marceau, N.; Blouin, M. J.; Germain, L., et al. Role of different epithelial cell types in liver ontogenesis, regeneration and neoplasia. In Vitro Cell. Devel. Biol. 25:336-341; 1989.
256. Fausto, N.; Mead, J. E. Regulation of liver growth: protooncogenes and transforming growth factors. Lab Invest. 60:4-13; 1989.
257. Wolff, J. A.; Yee, J. K.; Skelly, H. F., et al. Expression of retrovirally transduced genes in primary cultures of adult rat hepatocytes. Proc. Natl. Acad. Sci. USA 84:3344-3348; 1987.
258. Ledley, F. D.; Darlington, G. J.; Hahn, T., et al. Retroviral gene transfer into primary hepatocytes. Implications for genetic therapy of liver-specific functions. Proc. Natl. Acad. Sci. USA 84:5335-5339; 1987.
259. Wilson, J. M.; Johnston, D. E.; Jefferson, D. M., et al. Correction of the genetic defect in hepatocytes from the Watanabe heritable hyperlipidemic rabbit. Proc. Natl. Acad. Sci. USA 85:4421-4425; 1988.
260. Thompson, J. A.; Anderson, K. D.; DiPietro, J. M., et al. Site-directed neovessel formation in vivo. Science 241:1349-1352; 1988.
261. Bell, E.; Sher, S.; Hull, B., et al. The reconstitution of living skin. J. Invest. Dermatol. 81(1 Suppl):2s-10s; 1983.
262. Gallico, G. G.; O'Connor, N. E.; Compton, C. C., et al. Permanent coverage of large burn wounds with autologous cultured human epithelium. N. Engl. J. Med. 311:448-451; 1984.
263. Bell, E.; Moore, H.; Mitchie, C., et al. Reconstruction of a thyroid gland equivalent from cells and matrix materials. J. Exp. Zool. 232:277-285; 1984.
264. Weinberg, C. B.; Bell, E. A blood vessel model constructed from collagen and cultured vascular cells. Science 231:397-400; 1986.
265. Morgan, J. R.; Barrandon, Y.; Green, H., et al. Expression of an exogenous growth hormone gene by transplantable human epidermal cells. Science 237:1476-1479; 1987.
266. Zwiebel, J. A.; Freeman, S. M.; Kantoff, P. W., et al. High-level recombinant gene expression in rabbit endothelial cells transduced by retroviral vectors. Science 243:220-222; 1989.
267. Wilson, J. M.; Birinyi, L. K.; Saloman, R. N., et al. Implantation of vascular grafts lined with genetically modified endothelial cells. Science 244:1344-1346; 1989.

268. Thompson, J. A.; Haudenschild, C. C.; Anderson, K. D., et al. Induction of organoid neovascular structures in vivo. Proc. Natl. Acad. Sci., in press; 1989.
269. Thilly, B., ed. *Mammalian Cell Technology*, Butterworths & Co., London; 1986.
270. Griffiths, J. B.; Spear, R., eds. *Modern Approaches to Animal Cell Technology*, Butterworths & Co., London; 1987.
271. James, K.; Bell, G. T. Human monoclonal antibody production. J. Immunol. Methods 100:5–40; 1987.
272. Murakami, H. Serum-free media used for cultivation of hybridomas. In: Mizrahi, A., ed. *Monoclonal Antibodies: Production and Application*, Alan R. Liss, NY, 1989:107–113.

The authors are indebted to many colleagues whose personal communications and original published contributions to the concepts and developments reviewed here could not be cited due to space limitations. We thank Sydney Spraggs for editorial assistance.