CHAPTER 1

Introduction

As tissue culture enters its second century since its inception [Harrison, 1907], it is reaching what is probably one of, if not the, most exciting times in its history. For the first time it is possible for genetic manipulation of commonly and easily cultured cells, such as skin fibroblasts, to allow their conversion into pluripotent stem (iPS) cells, capable of differentiating into a range of different cell types [Lewitzky & Yamanaka, 2007; Nakagawa et al., 2007; Yu et al., 2007]. Coupled with the use of a chemical inducer of transcriptional changes in the genome (valproic acid), the four genes previously required is reduced to two [Huangfu et al., 2008] and the possibility of creating iPS cells by biochemical induction, rather than genetic intervention, becomes a real possibility. Added to that is the demonstration that it may also be possible to induce transdifferentiation from one lineage to another [Kondo & Raff, 2000; Le Douarin et al., 2004], and the field opens up to a whole new scenario: instead of the need for complex selective culture techniques, simple culture procedures may be used to initiate a cell line and biochemical regulation may be used to convert it into a new phenotype, directly via regression to a stem cell or to other progenitor cell. The possibilities that this opens up for the study of the regulation of differentiation, the determination of errors that occur in abnormal differentiation [Ebert et al., 2009] and malignancy, the provision of screening systems for diagnosis and drug development with cell lines from known pathologies, and the creation of autografts by tissue engineering promise a further expansion of tissue culture technology and usage comparable to the biotechnology boom of the turn of the century.

1.1 HISTORICAL BACKGROUND

Tissue culture was devised at the beginning of the twentieth century [Harrison, 1907; Carrel, 1912] (Table 1.1) as a method for studying the behavior of animal cells free of systemic variations that might arise in vivo both during normal homeostasis and under the stress of an experiment. As the name implies, the technique was elaborated first with undisaggregated fragments of tissue, and growth was restricted to the radial migration of cells from the tissue fragment, with occasional mitoses in the outgrowth. As culture of cells from and within such primary explants of tissue dominated the field for more than 50 years [Fischer, 1925; Parker, 1961], it is not surprising that the name ''tissue culture'' has remained in use as a generic term despite the fact that most of the explosive expansion in the field in the second half of the twentieth century (Fig. 1.1) was made possible by the use of dispersed cell cultures.

Disaggregation of explanted cells and subsequent plating out of the dispersed cells was first demonstrated by Rous [Rous & Jones, 1916], although passage was more often by surgical subdivision of the culture by Fischer, Carrel, and others, to generate what were then termed cell strains. L929 was the first cloned cell strain, isolated by capillary cloning from mouse L-cells [Sanford et al., 1948]. It was not until the 1950s that trypsin became more generally used for subculture, following procedures described by Dulbecco to obtain passaged monolayer cultures for viral plaque assays [Dulbecco, 1952], and the generation of a single cell suspension by trypsinization, which facilitated the further

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TABLE 1.1. Key Events in Development of Cell and Tissue Cultures

Date	Event	Reference
1907	Frog embryo nerve fiber outgrowth in vitro	Harrison, 1907
1912	Explants of chick connective tissue; heart muscle contractile for 2–3 months	Carrel, 1912; Burrows, 1912
1916	Trypsinization and subculture of explants	Rous & Jones, 1916
1923	Subculture of fibroblastic cell lines	Carrel & Ebeling, 1923
1925–26	Differentiation of embryonic tissues in organ culture	Strangeways & Fell, 1925, 1926
1929	Organ culture of chick long bones	Fell & Robison, 1929
1948	Introduction of use of antibiotics in tissue culture	Keilova, 1948; Cruikshank & Lowbury, 1952
1943	Establishment of the L-cell mouse fibroblast; first continuous cell line	Earle et al., 1943
1948	Cloning of the L-cell	Sanford et al., 1948
1949	Growth of virus in cell culture	Enders et al., 1949
1952	Use of trypsin for generation of replicate subcultures	Dulbecco, 1952
	Virus plaque assay	Dulbecco, 1952
	Salk polio vaccine grown in monkey kidney cells	Kew et al., 2005
	Establishment the first human cell line, HeLa, from a cervical carcinoma	Gey et al., 1952
1954	Fibroblast contact inhibition of cell motility	Abercrombie & Heaysman, 1953, 1954
1955	Cloning of HeLa on a homologous feeder layer	Puck & Marcus, 1955
	Development of defined media	Eagle, 1955, 1959
	Requirement of defined media for serum growth factors	Sanford et al., 1955; Harris, 1959
Late 1950s	Realization of importance of mycoplasma (PPLO) infection	Coriell et al., 1958; Rothblat & Morton, 1959; Nelson, 1960
	Nuclear transplantation	Briggs & King, 1960; Gurdon, 1960
1961	Definition of finite life span of normal human cells	Hayflick & Moorhead, 1961
	Cell fusion—somatic cell hybridization	Sorieul & Ephrussi, 1961
1962	Establishment and transformation of BHK21	Macpherson & Stoker, 1962
	Maintenance of differentiation (pituitary & adrenal tumors)	Buonassisi et al., 1962; Yasamura et al., 1966; Sato & Yasamura, 1966
1963	3T3 cells & spontaneous transformation	Todaro & Green, 1963
1964	Pluripotency of embryonal stem cells	Kleinsmith & Pierce, 1964
	Selection of transformed cells in agar	Macpherson & Montagnier, 1964
1964–69	Rabies, mumps, and Rubella vaccines in WI-38 human lung fibroblasts	Wiktor et al., 1964; Sokol et al., 1968
1965	Serum-free cloning of Chinese hamster cells	Ham, 1965
	Heterokaryons—man-mouse hybrids	Harris & Watkins, 1965
1966	Nerve growth factor	Levi-Montalcini, 1966
	Differentiation in rat hepatomas	Thompson et al., 1966
	Colony formation by hematopoietic cells	Bradley & Metcalf, 1966; Ichikawa et al., 1966
1967	Epidermal growth factor	Hoober & Cohen 1967
	HeLa cell cross-contamination	Gartler, 1967
	Density limitation of cell proliferation	Stoker & Rubin, 1967
	Lymphoblastoid cell lines	Moore et al., 1967; Gerper et al., 1969; Miller et al., 1971
1968	Retention of differentiation in cultured normal myoblasts	Yaffe, 1968
	Anchorage independent cell proliferation	Stoker et al, 1968
1969	Colony formation in hematopoietic cells	Metcalf, 1969; Metcalf, 1990
1970s	Development of laminar flow cabinets	Kruse et al., 1991; Collins & Kennedy, 1999
1973	DNA transfer, calcium phosphate	Graham & Van der Eb, 1973
1975	Growth factors	Gospodarowicz, 1974; Gospodarowicz & Moran, 1974

TABLE 1.1. (*Continued*)

Date	Event	Reference
	Hybridomas—monoclonal antibodies	Kohler & Milstein, 1975
1976	Totipotency of embryonal stem cells	Illmensee & Mintz, 1976
	Growth factor supplemented serum-free media	Hayashi & Sato, 1976
1977	Confirmation of HeLa cell cross-contamination of many cell lines	Nelson-Rees & Flandermeyer, 1977
	3T3 feeder layer and skin culture	Green, 1977
1978	MCDB selective, serum-free media	Ham & McKeehan, 1978
	Matrix interactions	Gospodarowicz et al., 1978b; Reid & Rojkind, 1979
	Cell shape and growth control	Folkman & Moscona, 1978
1980s	Regulation of gene expression	Darnell, 1982
	Oncogenes, malignancy, and transformation	Weinberg, 1989
1980	Matrix from EHS sarcoma (later Matrigel™)	Hassell et al., 1980
1983	Regulation of cell cycle; cyclin	Evans et al., 1983; Nurse 1990
	Immortalization by SV40	Huschtscha & Holliday, 1983
1980–87	Development of many specialized cell lines	Peehl & Ham, 1980; Hammond et al., 1984; Knedler & Ham, 1987
1983	Reconstituted skin cultures	Bell et al., 1983
1984	Production of recombinant tissue-type plasminogen activator in mammalian cells	Collen et al 1984
1990s	Industrial scale culture of transfected cells for production of biopharmaceuticals	Butler, 1991
1991	Culture of human adult mesenchymal stem cells	Caplan, 1991
1998	Tissue engineered cartilage	Aigner et al., 1998
1998	Culture of human embryonic stem cells	Thomson et al., 1998
2000	Human Genome Project—genomics, proteomics, genetic deficiencies, and expression errors	Dennis et al., 2001
2002	Exploitation of tissue engineering	Atala & Lanza, 2002; Vunjak-Novakovic & Freshney, 2006
2007	Reprogramming of adult cells to become pluripotent stem (iPS) cells	Yu et al. 2007
2008	Induction of iPS cells by reprogramming with valproic acid	Huangfu et al. 2008

Note: See also Pollack [1981].

development of single cell cloning. Gey established the first continuous human cell line, HeLa [Gey et al., 1952]; this was subsequently cloned by Puck [Puck & Marcus, 1955] when the concept of an X-irradiated feeder layer was introduced into cloning. Tissue culture became more widely used at this time because of the introduction of antibiotics, which facilitated long-term cell line propagation, although many people were already warning against continuous use and the associated risk of harboring cryptic, or antibiotic-resistant, contaminations [Parker, 1961]. The 1950s were also the years of the development of defined media [Morgan et al., 1950; Parker et al., 1954; Eagle, 1955, 1959; Waymouth, 1959], which led ultimately to the development of serum-free media [Ham, 1963, 1965] (see Section 9.6).

Throughout this book the term *tissue culture* is used as a generic term to include organ culture and cell culture. The term *organ culture* will always imply a three-dimensional culture of undisaggregated tissue retaining some or all of the histological features of the tissue in vivo. *Cell culture* refers to a culture derived from dispersed cells taken from original tissue, from a primary culture, or from a cell line or cell strain by enzymatic, mechanical, or chemical disaggregation. The term *histotypic culture* implies that cells have been reaggregated or grown to recreate a three-dimensional structure with tissue-like cell density, for example, by cultivation at high density in a filter well, by perfusion and overgrowth of a monolayer in a flask or dish, by reaggregation in suspension over agar or in real or simulated zero gravity, or by infiltration of a three-dimensional matrix such as collagen gel. *Organotypic* implies the same procedures but recombining cells of different lineages, such as epidermal keratinocytes in combined culture with dermal fibroblasts, in an attempt to generate a *tissue equivalent*.

Harrison [1907] chose the frog as his source of tissue, presumably because it was a cold-blooded animal, and consequently incubation was not required. Furthermore because tissue regeneration is more common in lower vertebrates, he perhaps felt that growth was more likely to

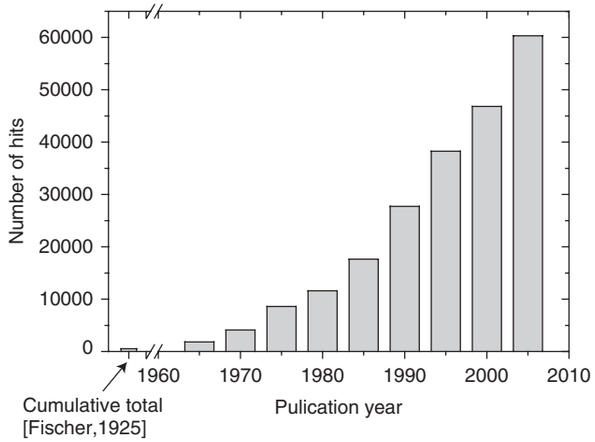

Fig. 1.1. Growth of Tissue Culture. Number of hits in PubMed for "cell culture" from 1965. The pre-1960 figure is derived from the bibliography of Fischer [1925].

occur than with mammalian tissue. Although his technique initiated a new wave of interest in the cultivation of tissue in vitro, few later workers were to follow his example in the selection of species. The stimulus from medical science carried future interest into warm-blooded animals, in which both normal development and pathological aberrations are closer to that found in humans. The accessibility of different tissues, many of which grew well in culture, made the embryonated hen's egg a favorite choice, but the development of experimental animal husbandry, particularly with genetically pure strains of rodents, brought mammals to the forefront as the favorite material. Although chick embryo tissue could provide a diversity of cell types in primary culture, rodent tissue had the advantage of producing continuous cell lines [Earle et al., 1943] and a considerable repertoire of transplantable tumors. The development of transgenic mouse technology [Beddington, 1992; Peat et al., 1992], together with the well-established genetic background of the mouse, has added further impetus to the selection of this animal as a favorite species.

The demonstration that human tumors could also give rise to continuous cell lines, such as HeLa [Gey et al., 1952], encouraged interest in human tissue, helped later by the classic studies of Leonard Hayflick on the finite life span of cells in culture [Hayflick & Moorhead, 1961] and the requirement of virologists and molecular geneticists to work with human material. The cultivation of human cells received a further stimulus when a number of different serum-free selective media were developed for specific cell types, such as epidermal keratinocytes, bronchial epithelium, and vascular endothelium (*see* Section 9.2.2). These formulations are now available commercially, although the cost remains high relative to the cost of regular media.

For many years the lower vertebrates and the invertebrates were largely ignored, although unique aspects of their development (tissue regeneration in amphibians, metamorphosis in insects) make them attractive systems for the study of the molecular basis of development. More recently the needs of agriculture and pest control have encouraged toxicity and virological studies in insects, and developments in gene technology have suggested that insect cell lines with baculovirus and other vectors may be useful producer cell lines because of the possibility of inserting larger genomic sequences in the viral DNA and a reduced risk of propagating human pathogenic viruses. Furthermore the economic importance of fish farming and the role of freshwater and marine pollution have stimulated more studies of normal development and pathogenesis in fish. Procedures for handling nonmammalian cells have tended to follow those developed for mammalian cell culture, although a limited number of specialized media are now commercially available for fish and insect cells (*see* Section 27.5).

The types of investigation that lend themselves particularly to tissue culture are summarized in Fig. 1.2. These include basic studies on cellular metabolism, the regulation of gene expression and the cell phenotype at different stages of development, and the application of these studies to immunology, pharmacology, toxicology, and tissue regeneration and transplantation.

Initially the development of cell culture owed much to the needs of two major branches of medical research: the production of antiviral vaccines and the understanding of neoplasia. The standardization of conditions and cell lines for the production and assay of viruses undoubtedly provided much impetus to the development of modern tissue culture technology, particularly the production of large numbers of cells suitable for biochemical and molecular analysis. This and other technical improvements made possible by the commercial supply of reliable media and sera and by the greater control of contamination with antibiotics and clean-air equipment have made tissue culture accessible to a wide range of interests. Tissue culture is no longer an esoteric interest of a few but a major research tool in many disciplines and a huge commercial enterprise.

An additional force of increasing weight from public opinion has been the expression of concern by many animal-rights groups over the unnecessary use of experimental animals. Although most accept the idea that some requirement for animals will continue for preclinical trials of new pharmaceuticals, there is widespread concern that extensive use of animals for cosmetics development and similar activities may not be morally justifiable. Hence there is an ever-increasing lobby for more in vitro assays. The adoption in vitro assays, however, still requires proper validation and general acceptance. Although this seemed a distant prospect some years ago, the introduction of more sensitive and specifically targeted in vitro assays, together with a very real prospect of assaying for inflammation in vitro, has promoted an unprecedented expansion of in vitro testing (see Section 21.4).

The introduction of cell fusion techniques (*see* Section 27.6) and genetic manipulation [Maniatis et al.,

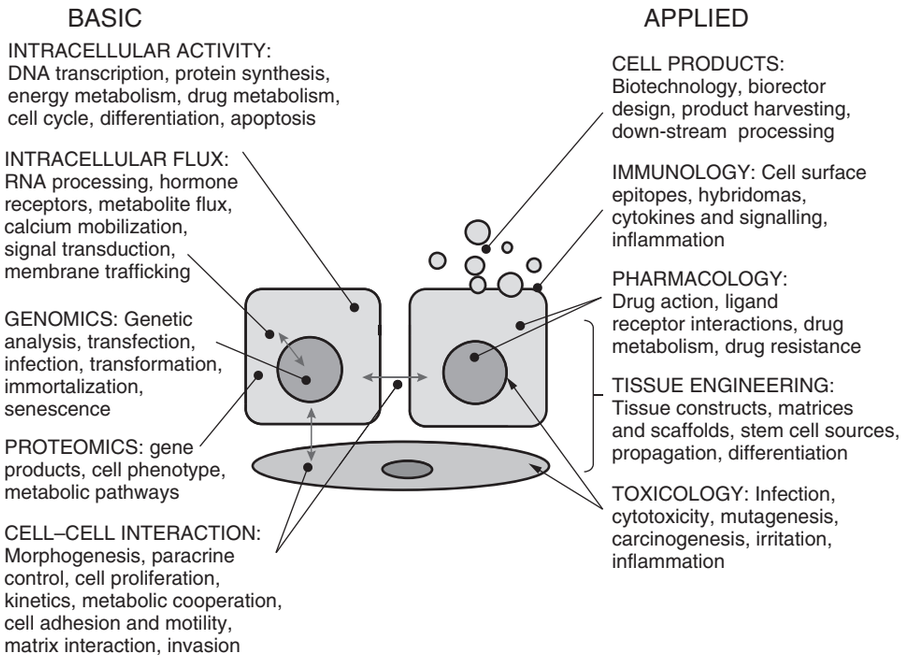

Fig. 1.2. Tissue Culture Applications.

1978; Shih et al., 1979] established somatic cell genetics as a major component in the genetic analysis of higher animals, including humans. The technology has expanded rapidly and now includes sophisticated procedures for DNA sequencing, and gene transfer, insertion, deletion, and silencing. This technology has led to a major improvement in our understanding of how the regulation of gene expression and protein synthesis influence the expression of the normal and abnormal phenotype. The entire human genome has been sequenced in the Human Genome Project [Baltimore, 2001], and a new dimension added to expression analysis with multigene array technology [Iyer et al., 1999].

The insight into the mechanism of action of antibodies and the reciprocal information that this provided about the structure of the epitope, derived from monoclonal antibody techniques [Kohler & Milstein, 1975], was, like the technique of cell fusion itself, a prologue to a whole new field of studies in genetic manipulation. A vast new technology and a multibillion-dollar industry have grown out of the ability to insert exploitable genes into prokaryotic and eukaryotic cells. Cell products such as human growth hormone, insulin, interferon, and many antibodies are now produced routinely by genetically modified cells. The absence of post-transcriptional modifications, such as glycosylation, in bacteria suggests that mammalian cells may provide more suitable vehicles [Grampp et al., 1992], particularly in light of developments in immortalization technology (*see* Section 17.4).

The study of cell interactions and cell signaling in cell differentiation and development [Jessell and Melton, 1992; Ohmichi et al., 1998; Balkovetz & Lipschutz, 1999] (*see also* Sections 2.2, 2.5, 16.7.1) have not only provided valuable fundamental information on mechanisms but have opened up whole new areas for tissue transplantation. Initial observations that cultures of epidermal cells form functionally differentiated sheets [Green et al., 1979] and endothelial cells may form capillaries [Folkman & Haudenschild, 1980] offered possibilities in homografting and reconstructive surgery using an individual's own cells [Limat et al., 1996; Tuszynski et al., 1996; Gustafson et al., 1998], particularly for severe burns [Gobet et al., 1997; Wright et al., 1998; Vunjak-Novakovic, 2006] (*see also* Section 25.4). With the ability to transfect normal genes into genetically deficient cells, it has become possible to graft such ''corrected'' cells back into the patient. Transfected cultures of rat bronchial epithelium carrying the β-gal reporter gene were shown to become incorporated into the rat's bronchial lining when they were introduced as an aerosol into the respiratory tract [Rosenfeld et al., 1992]. Similarly, cultured satellite cells were shown to be incorporated into wounded rat skeletal muscle, with nuclei from grafted cells appearing in mature, syncytial myotubes [Morgan et al., 1992]. Transfecting the normal insulin gene into β-islet cells cultured from diabetics, or even transfecting other cell types such as skeletal muscle progenitors [Morgan et al., 1992], would allow the cells to be incorporated into a low-turnover compartment and, potentially, give a long-lasting physiological benefit. Although the ethics of this type of approach seem less contentious, the technical limitations are still apparent.

Progress in neurological research has not had the benefit, however, of working with propagated cell lines from normal brain or nervous tissue, as the propagation of neurons in vitro has not been possible, until now, without resorting to the use of transformed cells (*see* Section 17.4). However, developments with human embryonal stem cell cultures [Thomson et al., 1998; Webber & Minger, 2004] suggest that this approach may provide replicating cultures that will differentiate into neurons and may provide useful and specific models for neuronal diseases [Ebert et al., 2008].

The prospect of transplantation of cultured cells has generated a whole new branch of culture, that of tissue engineering [Atala & Lanza, 2002; Vunjak-Novakovic & Freshney, 2006], encompassing the generation of tissue equivalents by organotypic culture (*see* Section 25.4), isolation and differentiation of human embryonal stem (ES) cells and adult totipotent stem cells such as mesenchymal stem cells (MSCs), gene transfer, materials science, construction and utilization of bioreactors, and transplantation technology. The technical barriers are steadily being overcome, bringing the ethical questions to the fore. The technical feasibility of implanting normal fetal neurons into patients with Parkinson disease has been demonstrated; society must now decide to what extent fetal material may be used for this purpose.

In vitro fertilization (IVF), developed from early experiments in embryo culture [Edwards, 1996], is now widely used [e.g., *see* Gardner & Lane, 2003] and has been accepted legally and ethically in many countries. The use of surplus embryos for research has also been accepted in some countries and will provide valuable material to further increase understanding of developmental processes and how to handle the cell lines generated. However, another area of development raising significant ethical debate is the generation of gametes in vitro from the culture of primordial germ cells isolated from testis and ovary [Dennis, 2003] or from ES cells. Oocytes have been cultured from embryonic mouse ovary and implanted, generating normal mice [Eppig, 1996; Obata et al., 2002], and spermatids have been cultured from newborn bull testes and cocultured with Sertoli cells [Lee et al., 2001]. Similar work with mouse testes generated spermatids that were used to fertilize mouse eggs, which developed into mature, fertile adults [Marh et al., 2003].

Tissue culture has also been used for diagnosis and toxicology. Amniocentesis (*see* Section 23.3.1) can reveal genetic disorders in the early embryo, although the polymerase chain reaction (PCR) and direct sampling are gradually replacing this, and the toxic effects of pharmaceutical compounds and potential environmental pollutants can be assayed in vitro (*see* Sections 22.3.1, 22.3.2, 22.4). In vitro toxicology has acquired greater importance in recent years due to changes in legislation regarding the usage of experimental animals, particularly in Europe.

1.2 ADVANTAGES OF TISSUE CULTURE

1.2.1 Control of the Environment

The two major advantages of tissue culture (Table 1.2) are the ability to control the physiochemical environment (pH, temperature, osmotic pressure, and O_2 and CO_2 tension), which has to be controlled very precisely, and the physiological conditions, which have to be kept relatively constant. However, the physiological environment cannot always be defined where cell lines still require supplementation of the medium with serum or other poorly defined constituents. These supplements are prone to batch variation and contain undefined elements such as hormones and other stimulants and inhibitors. The identification of some of the essential components of serum (*see* Table 8.5), together with a better understanding of factors regulating cell proliferation (*see* Table 9.4), has made the replacement of serum with defined constituents feasible (*see* Section 9.4). The role of the extracellular matrix (ECM) is important but similar to the use of serum—that is, the matrix is often necessary, but not always precisely defined. Prospects for defined ECM improve, however, as cloned matrix constituents become available [Kortesmaa et al., 2000; Belin & Rousselle, 2006; Braam et al., 2008; Dame & Verani, 2008; Domogatskaya et al., 2008] (see also Appendix II).

1.2.2 Characterization and Homogeneity of Samples

Tissue samples are invariably heterogeneous. Replicates, even from one tissue, vary in their constituent cell types. After one or two passages, cultured cell lines assume a homogeneous (or at least uniform) constitution, as the cells are randomly mixed at each transfer and the selective pressure of the culture conditions tends to produce a homogeneous culture of the most vigorous cell type. Hence, at each subculture, replicate samples are identical to each other, and the characteristics of the line may be perpetuated over several generations, or even indefinitely if the cell line is stored in liquid nitrogen. Because experimental replicates are virtually identical, the need for statistical analysis of variance is simplified. Furthermore the availability of stringent tests for cell line identity (*see* Section 15.4) and contamination (*see* Sections 12.1.1, 18.3, 18.6) means that preserved stocks may be validated for future research and commercial use.

1.2.3 Economy, Scale, and Mechanization

Cultures may be exposed directly to a reagent at a lower, and defined, concentration and with direct access to the cell. Consequently less reagent is required than for injection in vivo, where >90% may be lost by excretion and distribution to tissues other than those under study. Screening tests with many variables and replicates are cheaper, and the legal, moral, and ethical questions of animal experimentation are avoided. New developments in multiwell plates and robotics also have introduced significant economies in time and scale.

TABLE 1.2. Advantages of Tissue Culture

Category	Advantages
Physicochemical environment	Control of pH, temperature, osmolality, dissolved gases
Physiological conditions	Control of hormone & nutrient concentrations
Microenvironment	Regulation of matrix, cell-cell interaction, gaseous diffusion
Cell line homogeneity	Availability of selective media; cell cloning
Characterization	Easily performed cytology, DNA profiling, immunostaining
Preservation	Stocks stored in liquid nitrogen
Validation & accreditation	Origin, history, purity authenticated and recorded
Replicates and variability	Easy quantitation and minimal statistical analysis
Reagent saving	Reduced volumes, direct access to cells, lower cost
Control of $C \times T$	Ability to define dose, concentration, time
Mechanization	Available with microtitration and robotics
Scale	Number of replicates can be increased substantially
Time saving	Assay time reduced, at least, by an order of magnitude
Reduction of animal use	Cytotoxicity & screening of pharmaceutics, cosmetics, etc.

1.2.4 In vitro Modeling of In vivo Conditions

Perfusion techniques allow the delivery of specific experimental compounds to be regulated in concentration, duration of exposure (*see* Table 1.2), and metabolic state. The development of histotypic and organotypic models, with a more accurate replication of the in vivo cell phenotypes, also increases the accuracy of in vivo modeling.

1.3 LIMITATIONS

1.3.1 Expertise

Culture techniques must be carried out under strict aseptic conditions because animal cells grow much less rapidly than many of the common contaminants, such as bacteria, molds, and yeasts (Table 1.3). Furthermore, unlike microorganisms, cells from multicellular animals do not normally exist in isolation and consequently are not able to sustain an independent existence without the provision of a complex environment simulating blood plasma or interstitial fluid. These conditions imply a level of skill and understanding on the part of the operator in order to appreciate the requirements of the system and to diagnose problems as they arise (*see* Chapters 2, 33). Also care must be taken to avoid the recurrent problem of cross-contamination and to authenticate stocks (*see* Sections 12.1.1, 15.2, 18.6). Hence tissue culture should not be undertaken casually to run one or two experiments, but requires proper training (*see* Chapter 28), strict control of procedures, and a controlled environment.

TABLE 1.3. Limitations of Tissue Culture

Category	Examples
Necessary expertise	Sterile handling
	Avoidance of chemical contamination
	Detection of microbial contamination
	Awareness and detection of mis-identification
Environmental control	Isolation and cleanliness of workplace
	Incubation, pH control
	Containment and disposal of biohazards
Quantity and cost	Capital equipment for scale-up
	Medium, serum
	Disposable plastics
Genetic instability	Heterogeneity, variability
Phenotypic instability	Dedifferentiation
	Adaptation
	Selective overgrowth
Identification of cell type	Markers not always expressed
	Histology difficult to recreate and atypical
	Geometry and microenvironment changes cytology

1.3.2 Quantity

A major limitation of cell culture is the expenditure of effort and materials that goes into the production of relatively little tissue. A realistic maximum per batch for most small laboratories (with two or three people doing tissue culture) might be 1 to 10 g, wet weight, of cells. With a little more effort and the facilities of a larger laboratory, 10 to 100 g is possible; above 100 g implies industrial pilot-plant scale, a level that is beyond the reach of most laboratories but is not impossible if special facilities are provided, when kilogram quantities can be generated.

The cost of producing cells in culture is about 10 times that of using animal tissue. Consequently, if large amounts

of tissue (>10 g) are required, the reasons for providing them by culture must be very compelling. For smaller amounts of tissue (~10 g), the costs are more readily absorbed into routine expenditure, but it is always worth considering whether assays or preparative procedures can be scaled down. Microscale and nanoscale assays can often be quicker because of reduced manipulation times, volumes, and centrifuge times, for example, and so these assays are frequently more readily automated (*see* Sections 20.8, 21.3.5). Scaling down and automating assays enable more tests to be done, which in turn may require the cell preparation to be automated (*see* Section 26.4).

1.3.3 Dedifferentiation and Selection

When the first major advances in cell line propagation were achieved in the 1950s, many workers observed the loss of the phenotypic characteristics typical of the tissue from which the cells had been isolated. This effect was blamed on dedifferentiation, a process assumed to be the reversal of differentiation but later shown to be largely due to the overgrowth of undifferentiated cells of the same or a different lineage. The development of serum-free selective media (*see* Section 9.2.2) has now made the isolation of specific lineages possible, and it can be seen that under the right conditions, many of the differentiated properties of these cells may be restored (*see* Section 16.7).

1.3.4 Origin of Cells

If differentiated properties are lost, for whatever reason, it is difficult to relate the cultured cells to functional cells in the tissue from which they were derived. Stable markers are required for characterization of the cells (*see* Section 15.1); in addition the culture conditions may need to be modified so that these markers are expressed (*see* Sections 2.4, 16.7). Regrettably, many cell lines have been misidentified due to cross-contamination or errors in stock control in culture or in the freezer (*see* Sections 12.1.1, 15.2, 18.6). This makes it essential to have the technology, or access to it, to ensure authentication of each cell line that is used (*see* Section 15.2).

1.3.5 Instability

Instability is a major problem with many continuous cell lines, resulting from their unstable aneuploid chromosomal constitution. Even with short-term cultures of untransformed cells, heterogeneity in growth rate and the capacity to differentiate within the population can produce variability from one passage to the next (*see* Section 17.3).

1.4 MAJOR DIFFERENCES IN VITRO

Most of the differences in cell behavior between cultured cells and their counterparts in vivo stem from the dissociation of cells from a three-dimensional geometry and their propagation on a two-dimensional substrate. Specific cell interactions characteristic of the histology of the tissue are lost. As the growth fraction of the cell population increases, the cells spread out, become mobile, and, in many cases, start to proliferate. When a cell line forms, it may represent only one or two cell types, and many heterotypic cell–cell interactions are lost.

The culture environment also lacks the several systemic components involved in homeostatic regulation in vivo, principally those of the nervous and endocrine systems. Without this control, cellular metabolism may be more constant in vitro than in vivo, but may not be truly representative of the tissue from which the cells were derived. Recognition of this fact has led to the inclusion of a number of different hormones in culture media (*see* Sections 9.4.4, 10.4.3), and it seems likely that this trend will continue. The low oxygen tension due to the lack of oxygen transporter (hemoglobin) results in energy metabolism in vitro occurring largely by glycolysis; although the citric acid cycle is still functional, it plays a lesser role.

It is not difficult to find many more differences between the environmental conditions of a cell in vitro and in vivo (*see* Section 21.2), and this disparity has often led to tissue culture being regarded in a rather skeptical light. Still, although the existence of such differences cannot be denied, many specific pathways and specialized functions are expressed in culture, and as long as the limits of the model are appreciated, tissue culture can be a very valuable tool.

1.5 TYPES OF TISSUE CULTURE

There are three main methods of initiating a culture [Schaeffer, 1990] (*see* Fig. 1.3; Table 1.4; Appendix IV): (1) *Organ culture* implies that the architecture characteristic of the tissue in vivo is retained, at least in part, in the culture (*see* Section 25.2). Toward this end the tissue is cultured at the liquid–gas interface (on a raft, grid, or gel), which favors the retention of a spherical or three-dimensional shape. (2) In *primary explant culture* a fragment of tissue is placed at a glass (or plastic)–liquid interface, where, after attachment, migration is promoted in the plane of the solid substrate (*see* Section 11.3.1). (3) *Cell culture* implies that the tissue, or outgrowth from the primary explant, is dispersed (mechanically or enzymatically) into a cell suspension, which may then be cultured as an adherent monolayer on a solid substrate or as a suspension in the culture medium *(see* Sections 11.3, 12.4.5).

Because of the retention of histological interactions found in the tissue from which the culture was derived, organ cultures tend to retain the differentiated properties of that tissue. They do not grow rapidly (cell proliferation is limited to the periphery of the explant and is restricted mainly to embryonic tissue) and hence cannot be propagated; each experiment requires fresh explantations, which implies greater effort and poorer reproducibility of the sample than is

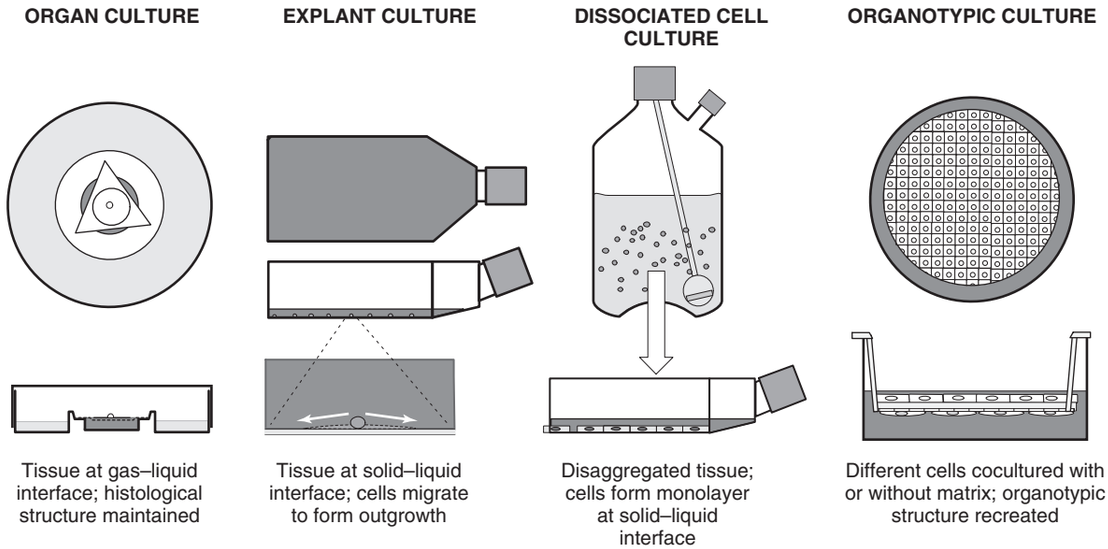

Fig. 1.3. Types of Tissue Culture.

TABLE 1.4. Properties of Different Types of Culture

Category	Organ culture	Explant	Cell culture	Organotypic culture
Source	Embryonic organs, adult tissue fragments	Tissue fragments	Disaggregated tissue, primary culture, propagated cell line	Primary culture or cell lines
Effort	High	Moderate	Low	Moderate
Characterization	Easy, by histology	Cytology and markers	Biochemical, molecular, immunological, and cytological assays	Histology, confocal microscopy, or MRI
Histology	Informative	Difficult	Not applicable	Informative
Biochemical differentiation	Possible	Heterogeneous	Lost, but may be reinduced	Often re-expressed
Propagation	Not possible	Possible from outgrowth	Standard procedure	Only after dissociation
Replicate sampling, reproducibility, homogeneity	High intersample variation	High intersample variation	Low intersample variation	Low intersample variation
Quantitation	Difficult	Difficult	Easy; many techniques available	May require image analysis

achieved with cell culture. Quantitation is therefore more difficult, and the amount of material that may be cultured is limited by the dimensions of the explant (~1 mm^3) and the effort required for dissection and setting up the culture.

Cell cultures may be derived from primary explants or dispersed cell suspensions. Because cell proliferation is often found in such cultures, the propagation of cell lines becomes feasible. A monolayer or cell suspension with a significant growth fraction (*see* Section 20.11.1) may be dispersed by enzymatic treatment or simple dilution and reseeded, or subcultured, into fresh vessels (Table 1.5; *see also* Sections 12.1, 12.4). This constitutes a *subculture* or *passage*, and the daughter cultures so formed are the beginnings of a *cell line*.

The formation of a cell line from a primary culture implies (1) an increase in the total number of cells over several generations (population doublings) and (2) the ultimate predominance of cells or cell lineages with a high proliferative

TABLE 1.5. Subculture

Advantages	Disadvantages
Propagation	Trauma of enzymatic or mechanical disaggregation
More cells	Selection of cells adapted to culture
Possibility of cloning	Overgrowth of unspecialized or stromal cells
Increased homogeneity	Genetic instability
Characterization of replicate samples	Loss of differentiated properties (may be inducible)
Frozen storage	Increased risk of misidentification or cross-contamination

capacity, resulting in (3) a degree of uniformity in the cell population (*see* Table 1.5). The line may be characterized, and the characteristics will apply for most of its finite life span. The derivation of *continuous* (or ''established,'' as they were once known) cell lines usually implies a genotypic change, or *transformation* (*see* Sections 3.8, 17.2), and the cell formation is usually accompanied by an increased rate of cell proliferation and a higher plating efficiency (*see* Section 17.5).

When cells are selected from a culture, by cloning or by some other method, the subline is known as a *cell strain*. A detailed characterization is then implied. Cell lines or cell strains may be propagated as an adherent monolayer or in suspension. *Monolayer* culture signifies that the cells are grown attached to the substrate. *Anchorage dependence* means that attachment to (and usually some degree of spreading onto) the substrate is a prerequisite for cell proliferation. Monolayer culture is the mode of culture common to most normal cells, with the exception of hematopoietic cells. *Suspension* cultures are derived from cells that can survive and proliferate without attachment *(anchorage independent);* this ability is restricted to hematopoietic cells, transformed cell lines, and transformed cells from malignant tumors. It can be shown, however, that a small proportion of cells that are capable of proliferation in suspension exists in many normal tissues (*see* Section 17.5.1). The identity of these cells remains unclear, but a relationship to the stem cell or uncommitted precursor cell compartment has been postulated. Cultured cell lines are more representative of precursor cell compartments in vivo than of fully differentiated cells, as most differentiated cells normally do not divide (*see* Sections 2.4, 16.3).

Because they may be propagated as a uniform cell suspension or monolayer, cell cultures have many advantages, in quantitation, characterization, and replicate sampling, but lack the retention of cell–cell interaction and cell–matrix interaction afforded by organ cultures. For this reason many workers have attempted to reconstitute three-dimensional cellular structures (*see* Sections 25.3, 25.4). Such developments have required the introduction, or at least redefinition, of certain terms. *Histotypic culture*, or *histoculture* (I use *histotypic culture)*, has come to mean the high-density, or ''tissue-like,'' culture of one cell type, whereas *organotypic* culture implies the presence of more than one cell type interacting, as the cells might, in the organ of origin. Organotypic culture has provided new prospects for the study of cell interaction among discrete, defined populations of homogeneous and potentially genetically and phenotypically defined cells and an opportunity to create differentiated populations of cells suitable for grafting.

In many ways some of the most exciting developments in tissue culture arise from recognizing the necessity of specific cell interaction in homogeneous or heterogeneous cell populations in culture. This recognition marks the transition from an era of fundamental molecular biology, in which many of the regulatory processes have been worked out at the cellular level, to an era of cell or tissue biology, in which that understanding is applied to integrated populations of cells, to a more precise elaboration of the signals transmitted among cells, and to the creation of fully functional tissues in vitro.