



Basic Principles of Cell Culture

R. Ian Freshney

Centre for Oncology and Applied Pharmacology, Cancer Research UK Beatson Laboratories, Garscube Estate, Bearsden, Glasgow G61 1BD, Scotland, UK, i.freshney@ntlworld.com

1. Introduction	4
2. Types of Cell Culture	4
2.1. Primary Explantation Versus Disaggregation	4
2.2. Proliferation Versus Differentiation	4
2.3. Organotypic Culture	7
2.4. Substrates and Matrices	9
3. Isolation of Cells for Culture	9
3.1. Tissue Collection and Transportation	9
3.2. Biosafety and Ethics	10
3.3. Record Keeping	11
3.4. Disaggregation and Primary Culture	11
4. Subculture	11
4.1. Life Span	12
4.2. Growth Cycle	12
4.3. Serial Subculture	14
5. Cryopreservation	14
6. Characterization and Validation	16
6.1. Cross-Contamination	16
6.2. Microbial Contamination	16
6.3. Characterization	18
6.4. Differentiation	18
Sources of Materials	20
References	21

Culture of Cells for Tissue Engineering, edited by Gordana Vunjak-Novakovic and R. Ian Freshney
Copyright © 2006 John Wiley & Sons, Inc.

I. INTRODUCTION

The bulk of the material presented in this book assumes background knowledge of the principles and basic procedures of cell and tissue culture. However, it is recognized that people enter a specialized field, such as tissue engineering, from many different disciplines and, for this reason, may not have had any formal training in cell culture. The objective of this chapter is to highlight those principles and procedures that have particular relevance to the use of cell culture in tissue engineering. Detailed protocols for most of these basic procedures are already published [Freshney, 2005] and will not be presented here; the emphasis will be more on underlying principles and their application to three-dimensional culture. Protocols specific to individual tissue types will be presented in subsequent chapters.

2. TYPES OF CELL CULTURE

2.1. Primary Explantation Versus Disaggregation

When cells are isolated from donor tissue, they may be maintained in a number of different ways. A simple small fragment of tissue that adheres to the growth surface, either spontaneously or aided by mechanical means, a plasma clot, or an extracellular matrix constituent, such as collagen, will usually give rise to an outgrowth of cells. This type of culture is known as a *primary explant*, and the cells migrating out are known as the *outgrowth* (Figs. 1.1, 1.2, See Color Plate 1). Cells in the outgrowth are selected, in the first instance, by their ability to migrate from the explant and subsequently, if subcultured, by their ability to proliferate. When a tissue sample is disaggregated, either mechanically or enzymatically (See Fig. 1.1), the suspension of cells and small aggregates that is generated will contain a proportion of cells capable of attachment to a solid substrate, forming a *monolayer*. Those cells within the monolayer that are capable of proliferation will then be selected at the first subculture and, as with the outgrowth from a primary explant, may give rise to a *cell line*. Tissue disaggregation is capable of generating larger cultures more rapidly than explant culture, but explant culture may still be preferable where only small fragments of tissue are available or the fragility of the cells precludes survival after disaggregation.,

2.2. Proliferation Versus Differentiation

Generally, the differentiated cells in a tissue have limited ability to proliferate. Therefore, differentiated cells do not contribute to the formation of a primary culture, unless special conditions are used to promote their attachment and preserve their differentiated status. Usually it is the proliferating committed precursor compartment of a tissue (Fig. 1.3), such as fibroblasts of the dermis or the basal epithelial layer of the epidermis, that gives rise to the bulk of the cells in a

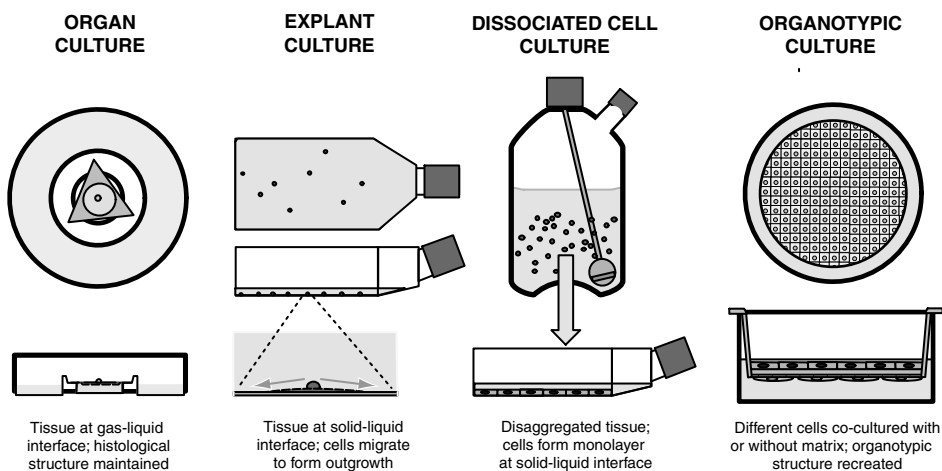


Figure 1.1. Types of culture. Different modes of culture are represented from left to right. First, an organ culture on a filter disk on a triangular stainless steel grid over a well of medium, seen in section in the lower diagram. Second, explant cultures in a flask, with section below and with an enlarged detail in section in the lowest diagram, showing the explant and radial outgrowth under the arrows. Third, a stirred vessel with an enzymatic disaggregation generating a cell suspension seeded as a monolayer in the lower diagram. Fourth, a filter well showing an array of cells, seen in section in the lower diagram, combined with matrix and stromal cells. [From Freshney, 2005.]

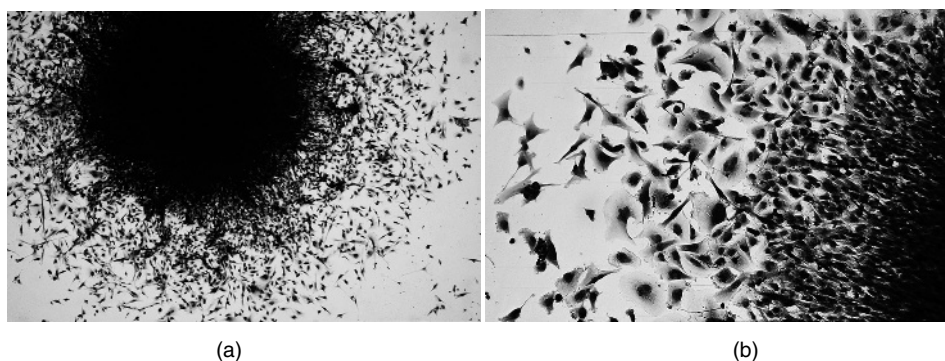


Figure 1.2. Primary explant and outgrowth. Microphotographs of a Giemsa-stained primary explant from human non-small cell lung carcinoma. a) Low-power (4× objective) photograph of explant (top left) and radial outgrowth. b) Higher-power detail (10× objective) showing the center of the explant to the right and the outgrowth to the left. (See Color Plate 1.)

primary culture, as, numerically, these cells represent the largest compartment of proliferating, or potentially proliferating, cells. However, it is now clear that many tissues contain a small population of regenerative cells which, given the correct selective conditions, will also provide a satisfactory primary culture, which may be propagated as stem cells or mature down one of several pathways toward

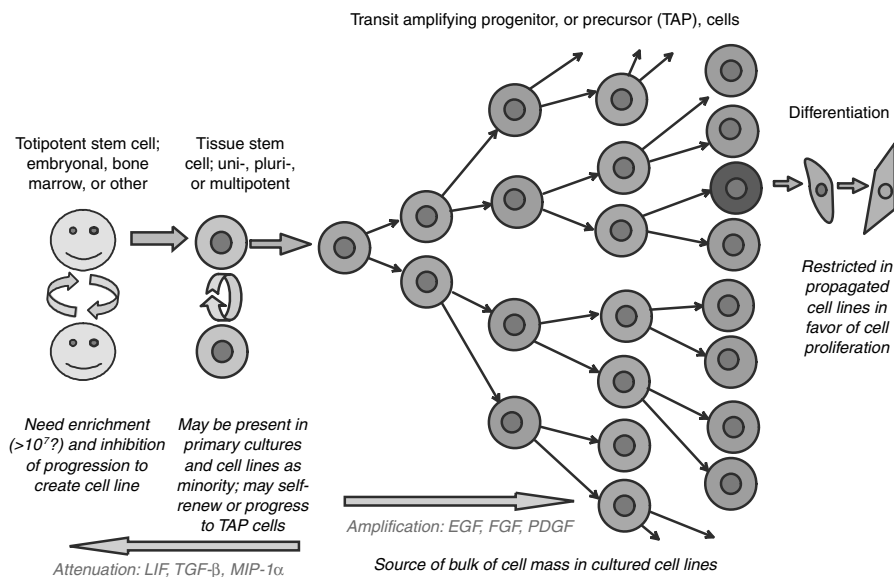


Figure 1.3. Origin of cell lines. Diagrammatic representation of progression from totipotent stem cell, through tissue stem cell (single or multiple lineage committed) to transit amplifying progenitor cell compartment. Exit from this compartment to the differentiated cell pool (far right) is limited by the pressure on the progenitor compartment to proliferate. Italicized text suggests fate of cells in culture and indicates that the bulk of cultured cells probably derive from the progenitor cell compartment, because of their capacity to replicate, but accepts that stem cells may be present but will need a favorable growth factor environment to become a significant proportion of the cells in the culture. [From Freshney, 2005.]

differentiation. This implies that not only must the correct population of cells be isolated, but the correct conditions must be defined to maintain the cells at an appropriate stage in maturation to retain their proliferative capacity if expansion of the population is required. This was achieved fortuitously in early culture of fibroblasts by the inclusion of serum that contained growth factors, such as platelet-derived growth factor (PDGF), that helped to maintain the proliferative precursor phenotype. However, this was not true of epithelial cells in general, where serum growth factors such as transforming growth factor β (TGF- β) inhibited epithelial proliferation and favored differentiation. It was not until serum-free media were developed [Ham and McKeehan, 1978, Mather, 1998, Karmioli, 2000] that this effect could be minimized and factors positive to epithelial proliferation, such as epidermal growth factor and cholera toxin, used to maximum effect.

Although undifferentiated precursors may give the best opportunity for expansion *in vitro*, transplantation may require that the cells be differentiated or carry the potential to differentiate. Hence, two sets of conditions may need to be used, one for expansion and one for differentiation. The factors required to induce differentiation will be discussed later in this chapter (See Section 7.4) and in later chapters. In general, it can be said that differentiation will probably require a selective medium for the cell type, supplemented with factors that favor differentiation, such as

retinoids, hydrocortisone, and planar-polar compounds, such as sodium butyrate (NaBt). In addition, the correct matrix interaction, homotypic and heterotypic cell interaction, and, for epithelial cells, the correct cellular polarity will need to be established, usually by using an organotypic culture. This assumes, of course, that tissue replacement will require the graft to be completely or almost completely differentiated, as is likely to be the case where extensive tissue repair is carried out. However, there is also the option that cell culture will only be required to expand a precursor cell type and the process of implantation itself will then induce differentiation, as appears to be the case with stem cell transplantation [Greco and Lecht, 2003].

2.3. Organotypic Culture

Dispersed cell cultures clearly lose their histologic characteristics after disaggregation and, although cells within a primary explant may retain some of the histology of the tissue, this will soon be lost because of flattening of the explant with cell migration and some degree of central necrosis due to poor oxygenation. Retention of histologic structure, and its associated differentiated properties, may be enhanced at the air/medium interface, where gas exchange is optimized and cell migration minimized, as distinct from the substrate/medium interface, where dispersed cell cultures and primary outgrowths are maintained. This so-called *organ culture* (See Fig. 1.1) will survive for up to 3 weeks, normally, but cannot be propagated. An alternative approach, with particular relevance to tissue engineering, is the amplification of the cell stock by generation of cell lines from specific cell types and their subsequent recombination in *organotypic culture*. This allows the synthesis of a tissue equivalent or construct on demand for basic studies on cell-cell and cell-matrix interaction and for in vivo implantation. The fidelity of the construct in terms of its real tissue equivalence naturally depends on identification of all the participating cell types in the tissue in vivo and the ability to culture and recombine them in the correct proportions with the correct matrix and juxtaposition. So far this has worked best for skin [Michel et al., 1999, Schaller et al., 2002], but even then, melanocytes have only recently been added to the construct, and islet of Langerhans cells are still absent, as are sweat glands and hair follicles, although some progress has been made in this area [Regnier et al., 1997; Laning et al., 1999].

There are a great many ways in which cells have been recombined to try to simulate tissue, ranging from simply allowing the cells to multilayer by perfusing a monolayer [Kruse et al., 1970] to highly complex perfused membrane (Membroferm [Klement et al., 1987]) or capillary beds [Knazek et al., 1972]. These are termed *histotypic cultures* and aim to attain the density of cells found in the tissue from which the cells were derived (Fig. 1.4). It is possible, using selective media, cloning, or physical separation methods (See Section 3.4), to isolate purified cell strains from disaggregated tissue or primary culture or at first subculture. These purified cell populations can then be combined in organotypic culture to recreate both the tissue cell density and, hopefully, the cell interactions and

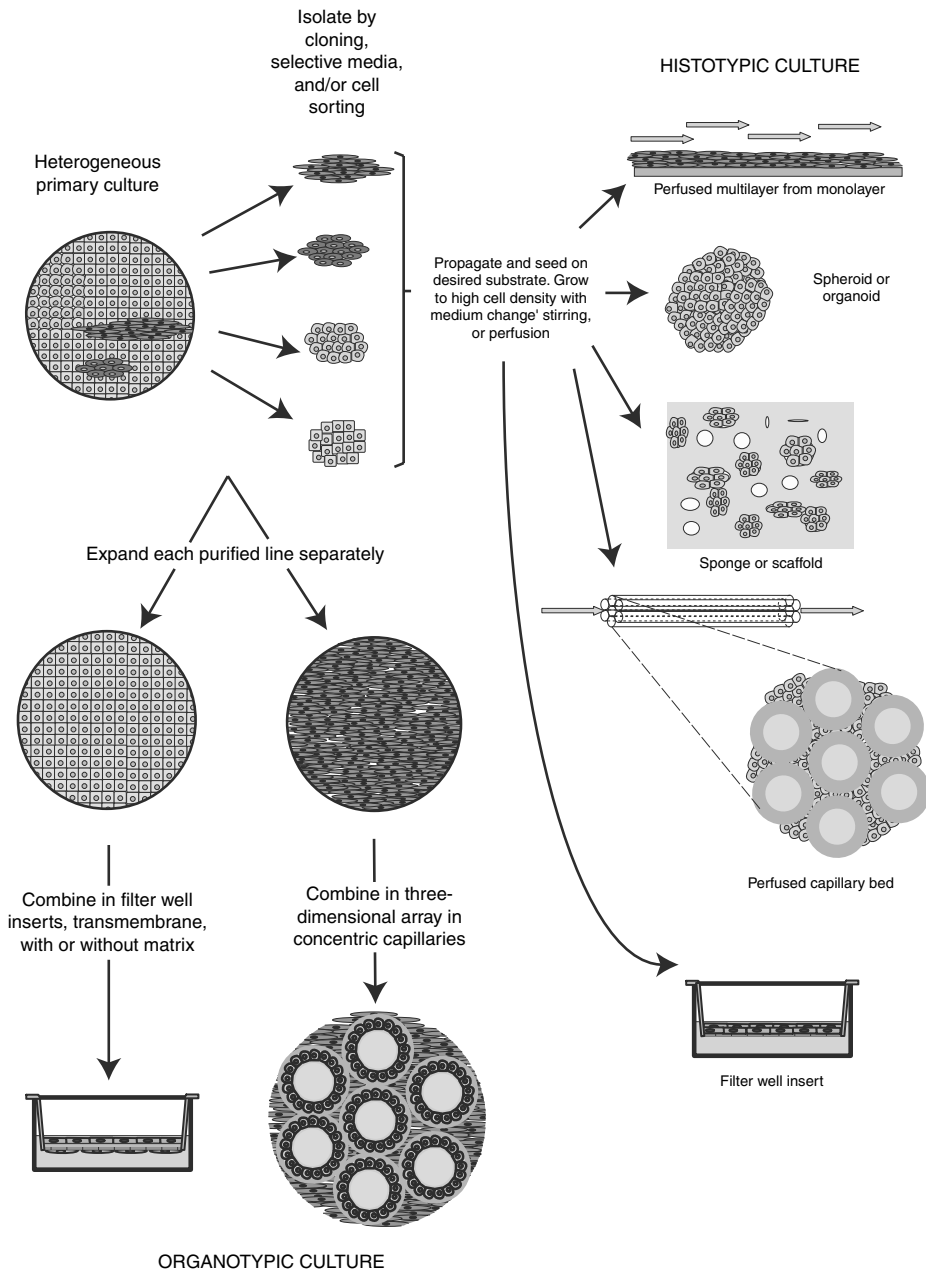


Figure 1.4. Histotypic and organotypic culture. Indicates the heterogeneity of a primary culture (top left), how this might be purified to give defined cell populations, which, if expanded and seeded into appropriate conditions can give high-density cultures of one cell type in perfused multilayers (top right), spheroids or organoids in stirred suspension (second top right), three-dimensional multilayers in perfused capillaries (third top right), or monolayers or multilayers in filter well inserts (bottom right). Expansion of purified populations and recombination can generate organotypic cultures, in filter well inserts (bottom left) or on concentric microcapillaries (bottom center). This last seems to be suggested by the architecture of the device (CellGro Triac), but the author has no knowledge of its use in this capacity.

matrix generation found in the tissue (See Fig. 1.4). Filter well inserts provide the simplest model system to test such recombinants, but there are many other possibilities including porous matrices, perfused membranes, and concentric double microcapillaries (Triac hollow fiber modules, [www.spectrapor.com/1/1/9.html]).

2.4. Substrates and Matrices

Initially, cultures were prepared on glass for ease of observation, but cells may be made to grow on many different charged surfaces including metals and many polymers. Traditionally, a net negative charge was preferred, such as found on acid-washed glass or polystyrene treated by electric ion discharge, but some plastics are also available with a net positive charge (e.g., Falcon Primaria), which is claimed to add some cell selectivity. In either case, it is unlikely that the cell attaches directly to synthetic substrates and more likely that the cell secretes matrix products that adhere to the substrate and provide ligands for the interaction of matrix receptors such as integrins. Hence it is a logical step to treat the substrate with a matrix product, such as collagen type IV, fibronectin, or laminin, to promote the adhesion of cells that would otherwise not attach.

The subject of scaffolds will be dealt with in detail in later chapters (See Part II). Suffice it to say at this stage that scaffolds have the same requirements as conventional substrates in terms of low toxicity and ability to promote cell adhesion, often with the additional requirement of a three-dimensional geometry. If the polymer or other material does not have these properties, derivatization and/or matrix coating will be required.

Most studies suggest that cell cultivation on a three-dimensional scaffold is essential for promoting orderly regeneration of engineered tissues *in vivo* and *in vitro*. Scaffolds investigated to date vary with respect to material chemistry (e.g., collagen, synthetic polymers), geometry (e.g., gels, fibrous meshes, porous sponges, tubes), structure (e.g., porosity, distribution, orientation, and connectivity of the pores), physical properties (e.g., compressive stiffness, elasticity, conductivity, hydraulic permeability), and degradation (rate, pattern, products).

In general, scaffolds should be made of biocompatible materials, preferentially those already approved for clinical use. Scaffold structure determines the transport of nutrients, metabolites, and regulatory molecules to and from the cells, whereas the scaffold chemistry may have an important role in cell attachment and differentiation. The scaffold should biodegrade at the same rate as the rate of tissue assembly and without toxic or inhibitory products. Mechanical properties of the scaffold should ideally match those of the native tissue being replaced, and the mechanical integrity should be maintained as long as necessary for the new tissue to mature and integrate.

3. ISOLATION OF CELLS FOR CULTURE

3.1. Tissue Collection and Transportation

The first, and most important, element in the collection of tissue is the cooperation and collaboration of the clinical staff. This is best achieved if a member of

the surgical team is also a member of the culture project, but even in the absence of this, time and care must be spent to ensure the sympathy and understanding of those who will provide the clinical material. It is worth preparing a short handout explaining the objectives of the project and spending some time with the person likely to be most closely involved with obtaining samples. This may be the chief surgeon (who will need to be informed anyway), or it may be a more junior member of the team willing to set up a collaboration, one of the nursing staff, or the pathologist, who may also require part of the tissue. Whoever fulfils this role should be identified and provided with labeled containers of culture medium containing antibiotics, bearing a contact name and phone number for the cell culture laboratory. A refrigerator should be identified where the containers can be stored, and the label should also state clearly **DO NOT FREEZE!** The next step is best carried out by someone from the laboratory collecting the sample personally, but it is also possible to leave instructions for transportation by taxi or courier. If a third party is involved, it is important to ensure that the container is well protected [See, for example, www.ehs.ucsf.edu/Safety%20Updates/Bsu/Bsu5.pdf], preferably double wrapped in a sealed polythene bag and an outer padded envelope provided with the name, address, and phone number of the recipient at the laboratory. Refrigeration during transport is not usually necessary, as long as the sample is not allowed to get too warm, but if delivery will take more than an hour or two, then one or two refrigeration packs, such as used in picnic chillers, should be included but kept out of direct contact.

If the tissue sample is quite small, a further tissue sample (any tissue) or a blood sample should be obtained for freezing. This will be used ultimately to corroborate the origin of any cell line that is derived from the sample by DNA profiling. A cell line is the culture that is produced from subculture of the primary, and every additional subculture after this increases the possibility of cross-contamination, so verification of origin is important (See Section 6). In addition, the possibility of misidentification arises during routine subculture and after recovery from cryopreservation (See Section 5).

3.2. Biosafety and Ethics

All procedures involved in the collection of human material for culture must be passed by the relevant hospital ethics committee. A form will be required for the patient to sign authorizing research use of the tissue, and preferably disclaiming any ownership of any materials derived from the tissue [Freshney, 2002, 2005]. The form should have a brief layman's description of the objectives of the work and the name of the lead scientist on the project. The donor should be provided with a copy.

All human material should be regarded as potentially infected and treated with caution. Samples should be transported securely in double-wrapped waterproof containers; they and derived cultures should be handled in a Class II biosafety cabinet and all discarded materials autoclaved, incinerated, or chemically disinfected. Each laboratory will its own biosafety regulations that should be adhered to, and anyone in any doubt about handling procedures should contact the local

safety committee (and if there is not one, create it!). Rules and regulations vary among institutions and countries, so it is difficult to generalize, but a good review can be obtained in Caputo [1996].

3.3. Record Keeping

When the sample arrives at the laboratory, it should be entered into a record system and assigned a number. This record should contain the details of the donor, identified by hospital number rather than by name, tissue site, and all information regarding collection medium, time in transit, treatment on arrival, primary disaggregation, and culture details, etc. [Freshney, 2002, 2005]. This information will be important in the comparison of the success of individual cultures, and if a long-term cell line is derived from the culture, this will be the first element in the cell line's provenance, which will be supplemented with each successive manipulation or experimental procedure. Such records are best maintained in a computer database where each record can be derived from duplication of the previous record with appropriate modifications. There may be issues of data protection and patient confidentiality to be dealt with when obtaining ethical consent.

3.4. Disaggregation and Primary Culture

Detailed information on disaggregation as a method for obtaining cells is provided in the appropriate chapters. Briefly, the tissue will go through stages of rinsing, dissection, and either mechanical disaggregation or enzymatic digestion in trypsin and/or collagenase. It is often desirable not to have a complete single-cell suspension, and many primary cells survive better in small clusters. Disaggregated tissue will contain a variety of different cell types, and it may be necessary to go through a separation technique [See Chapter 15, Freshney 2005], such as density gradient separation [Pretlow and Pretlow, 1989] or immunosorting by magnetizable beads (MACS), using a positive sort to select cells of interest [Carr et al., 1999] or a negative sort to eliminate those that are not required [Saalbach et al., 1997], or by using fluorescence-activated cell sorting (FACS) [See, e.g. Swope et al., 1997]. The cell population can then be further enriched by selection of the correct medium (e.g., keratinocyte growth medium (KGM) or MCDB 153 for keratinocytes [Peehl and Ham, 1980]), many of which are now available commercially (See Sources of Materials), and supplementing this with growth factors. Survival and enrichment may be improved in some cases by coating the substrate with gelatin, collagen, laminin, or fibronectin [Freshney, 2005].

4. SUBCULTURE

Frequently, the number of cells obtained at primary culture may be insufficient to create constructs suitable for grafting. Subculture gives the opportunity to expand the cell population, apply further selective pressure with a selective medium, and achieve a higher growth fraction and allows the generation of replicate cultures for

characterization, preservation by freezing, and experimentation. Briefly, subculture involves the dissociation of the cells from each other and the substrate to generate a single-cell suspension that can be quantified. Re seeding this cell suspension at a reduced concentration into a flask or dish generates a secondary culture, which can be grown up and subcultured again to give a tertiary culture, and so on. In most cases, cultures dedifferentiate during serial passaging but can be induced to redifferentiate by cultivation on a 3D scaffold in the presence of tissue-specific differentiation factors (e.g., growth factors, physical stimuli). However, the cell's ability to redifferentiate decreases with passaging. It is thus essential to determine, for each cell type, source, and application, a suitable number of passages during subculture. Protocols for subculture of specific cell types are given in later chapters, and a more general protocol is available in Chapter 13, Freshney [2005].

4.1. Life Span

Most normal cell lines will undergo a limited number of subcultures, or passages, and are referred to as *finite cell lines*. The limit is determined by the number of doublings that the cell population can go through before it stops growing because of senescence. Senescence is determined by a number of intrinsic factors regulating cell cycle, such as Rb and p53 [Munger and Howley, 2002], and is accompanied by shortening of the telomeres on the chromosomes [Wright and Shay, 2002]. Once the telomeres reach a critical minimum length, the cell can no longer divide. Telomere length is maintained by telomerase, which is downregulated in most normal cells except germ cells. It can also be higher in stem cells, allowing them to go through a much greater number of doublings and avoid senescence. Transfection of the telomerase gene hTERT into normal cells with a finite life span allows a small proportion of the cells to become immortal [Bodnar et al., 1998; Protocol 18.2, Freshney, 2005], although this probably involves deletion or inactivation of other genes such as p53 and *myc* [Cerni, 2000].

4.2. Growth Cycle

Each time that a cell line is subcultured it will grow back to the cell density that existed before subculture (within the limits of its finite life span). This process can be described by plotting a growth curve from samples taken at intervals throughout the growth cycle (Fig. 1.5), which shows that the cells enter a latent period of no growth, called the *lag period*, immediately after re seeding. This period lasts from a few hours up to 48 h, but is usually around 12–24 h, and allows the cells to recover from trypsinization, reconstruct their cytoskeleton, secrete matrix to aid attachment, and spread out on the substrate, enabling them to reenter cell cycle. They then enter exponential growth in what is known as the *log phase*, during which the cell population doubles over a definable period, known as the *doubling time* and characteristic for each cell line. As the cell population becomes crowded when all of the substrate is occupied, the cells become packed, spread less on the substrate, and eventually withdraw from the cell cycle. They then enter the *plateau* or *stationary phase*, where the growth fraction drops to close to zero. Some cells may

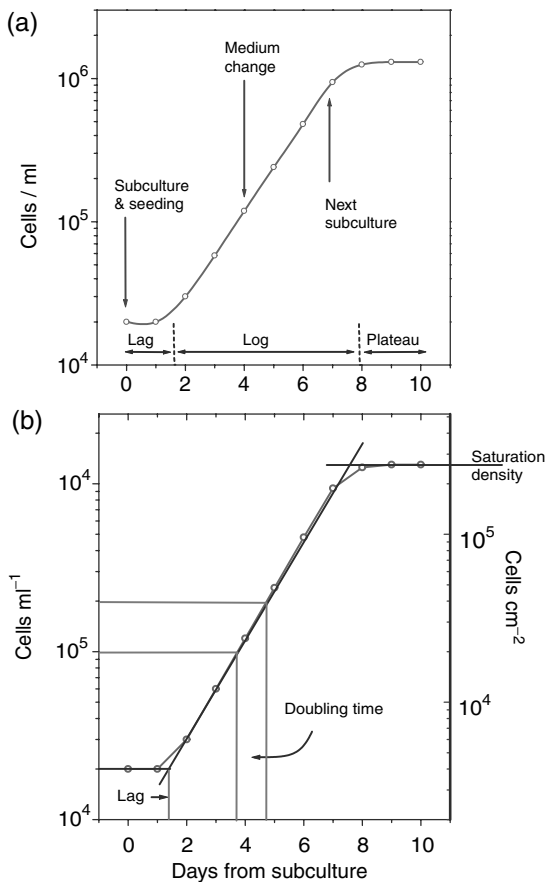


Figure 1.5. Growth curve. Increase in cell number on a log scale plotted against days from subculture. a) Defines the lag, log (exponential), and plateau phases, and when culture should be fed and subcultured after the indicated seeding time. b) Shows the kinetic parameters that can be derived from the growth curve: *lag* from the intercept between a line drawn through the points on the exponential phase and the horizontal from the seeding concentration; *doubling time* from the time taken, in the middle of the exponential phase, for the cell population to double; *saturation density* from the maximum (stable) cell density achieved by the culture, under the prevailing culture conditions. This is determined in cells/cm² (cell density rather than cell concentration) and is not absolute, as it will vary with culture conditions. It is best determined (as characteristic of the cell type) in conditions that are nonlimiting for medium, e.g., a small area of high-density cells in a large reservoir of medium (such as a coverslip, or a filter well insert, in a non-tissue culture-grade dish) or under continuous perfusion of medium. [Adapted from Freshney, 2005.]

differentiate in this phase; others simply exit the cell cycle into G₀ but retain viability. Cells may be subcultured from plateau, but it is preferable to subculture before plateau is reached, as the growth fraction will be higher and the recovery time (lag period) will be shorter if the cells are harvested from the top end of the log phase.

Reduced proliferation in the stationary phase is due partly to reduced spreading at high *cell density* and partly to exhaustion of growth factors in the medium at high *cell concentration*. These two terms are not interchangeable. Density implies that the cells are attached, and may relate to monolayer density (two-dimensional)

or multilayer density (three-dimensional). In each case there are major changes in cell shape, cell surface, and extracellular matrix, all of which will have significant effects on cell proliferation and differentiation. A high density will also limit nutrient perfusion and create local exhaustion of peptide growth factors [Stoker, 1973; Westermarck and Wasteson, 1975]. In normal cell populations this leads to a withdrawal from the cycle, whereas in transformed cells, cell cycle arrest is much less effective and the cells tend to enter apoptosis.

Cell concentration, as opposed to cell density, will exert its main effect through nutrient and growth factor depletion, but in stirred suspensions cell contact-mediated effects are minimal, except where cells are grown as aggregates. Cell concentration per se, without cell interaction, will not influence proliferation, other than by the effect of nutrient and growth factor depletion. High cell concentrations can also lead to apoptosis in transformed cells in suspension, notably in myelomas and hybridomas, but in the absence of cell contact signaling this is presumably a reflection of nutrient deprivation.

4.3. Serial Subculture

Each time the culture is subcultured the growth cycle is repeated. The number of doublings should be recorded (Fig. 1.6) with each subculture, simplified by reducing the cell concentration at subculture by a power of two, the so-called *split ratio*. A split ratio of two allows one doubling per passage, four, two doublings, eight, three doublings, and so on (See Fig. 1.6). The number of elapsed doublings should be recorded so that the time to senescence (See Section 4.1) can be predicted and new stock prepared from the freezer before the senescence of the existing culture occurs.

5. CRYOPRESERVATION

If a cell line can be expanded sufficiently, preservation of cells by freezing will allow secure stocks to be maintained without aging and protect them from problems of contamination, incubator failure, or medium and serum crises. Ideally, 1×10^6 – 1×10^7 cells should be frozen in 10 ampoules, but smaller stocks can be used if a surplus is not available. The normal procedure is to freeze a token stock of one to three ampoules as soon as surplus cells are available, then to expand remaining cultures to confirm the identity of the cells and absence of contamination, and freeze down a seed stock of 10–20 ampoules. One ampoule, thawed from this stock, can then be used to generate a using stock. In many cases, there may not be sufficient doublings available to expand the stock as much as this, but it is worth saving some as frozen stock, no matter how little, although survival will tend to decrease below 1×10^6 cells/ml and may not be possible below 1×10^5 cells/ml.

Factors favoring good survival after freezing and thawing are:

- (i) High cell density at freezing (1×10^6 – 1×10^7 cells/ml).
- (ii) Presence of a preservative, such as glycerol or dimethyl sulfoxide (DMSO) at 5–10%.

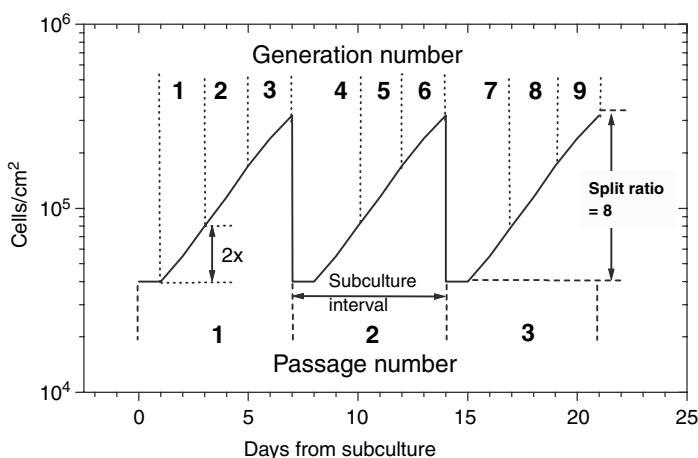


Figure 1.6. Serial subculture. Recurring growth curves during serial subculture, not necessarily recorded by daily cell counts, but predicted from one or two detailed growth curve analyses of these cells. Each cycle should be a replicate of the previous one, such that the same terminal cell density is achieved after subculture at the same seeding concentration. The lower number represents the passage number, i.e., the number of times the culture has been subcultured. The upper numbers represent the generation number, i.e., the number of times the population has doubled. In this example, the cell population doubles three times between each subculture, suggesting that the culture should be split 1:8 to regain the same seeding concentration each time. [From Freshney, 2005.]

- (iii) Slow cooling, 1 °C/min, down to -70°C and then rapid transfer to a liquid nitrogen freezer.
- (iv) Rapid thawing.
- (v) Slow dilution, ~ 20 -fold, in medium to dilute out the preservative.
- (vi) Reseeding at 2- to 5-fold the normal seeding concentration. For example, if cells are frozen at 5×10^6 cells in 1 ml of freezing medium with 10% DMSO and then thawed and diluted 1:20, the cell concentration will still be 2.5×10^5 cells/ml at seeding, higher than the normal seeding concentration for most cell lines, and the DMSO concentration will be reduced to 0.5%, which most cells will tolerate for 24 h.
- (vii) Changing medium the following day (or as soon as all the cells have attached) to remove preservative. Where cells are more sensitive to the preservative, they may be centrifuged after slow dilution and resuspended in fresh medium, but this step should be avoided if possible as centrifugation itself may be damaging to freshly thawed cells.

There are differences of opinion regarding some of the conditions for freezing and thawing, for example, whether cells should be chilled when DMSO is added or diluted rapidly on thawing, both to avoid potential DMSO toxicity. In the author's experience, chilling diminishes the effect of the preservative, particularly with glycerol, and rapid dilution reduces survival, probably due to osmotic shock. Culturing in diluted DMSO after thawing can be a problem for some cell lines if they

respond to the differentiating effects of DMSO, for example, myeloid leukemia cells, neuroblastoma cells, and embryonal stem cells; in these cases it is preferable to centrifuge after slow dilution at thawing or use glycerol as a preservative.

6. CHARACTERIZATION AND VALIDATION

6.1. Cross-Contamination

There has been much publicity about the very real risks of cross-contamination when handling cell lines [Marcovic and Marcovic, 1998; Macleod et al., 1999; Masters et al., 2001; van Bokhoven et al., 2001; Masters, 2002], particularly continuous cell lines. This is less of a problem with short-term cultures, but the risk remains that if there are other cell lines in use in the laboratory, they can cross-contaminate even a primary culture, or misidentification can arise during subculture or recovery from the freezer. If a laboratory focuses on one particular human cell type, superficial observation of lineage characteristics will be inadequate to ensure the identity of each line cultured. Precautions must be taken to avoid cross-contamination:

- (i) Do not handle more than one cell line at a time, or, if this is impractical, do not have culture vessels and medium bottles for more than one cell line open at one time, and never be tempted to use the same pipette or other device for different cell lines.
- (ii) Do not share media or other reagents among different cell lines.
- (iii) Do not share media or reagents with other people.
- (iv) Ensure that any spillage is mopped up immediately and the area swabbed with 70% alcohol.
- (v) Retain a tissue or blood sample from each donor and confirm the identity of each cell line by DNA profiling: (a) when seed stocks are frozen, (b) before the cell line is used for experimental work or transplantation.
- (vi) Keep a panel of photographs of each cell line, at low and high densities, above the microscope, and consult this regularly when examining cells during maintenance. This is particularly important if cells are handled over an extended period, and by more than one operator.
- (vii) If continuous cell lines are in use in the laboratory, handle them after handling other, slower-growing, finite cell lines.

6.2. Microbial Contamination

Antibiotics are often used during collection, transportation, and dissection of biopsy samples because of the intrinsic contamination risk of these operations. However, once the primary culture is established, it is desirable to eliminate

antibiotics as soon as possible. If the culture grows well, then antibiotics can be removed from the bulk of the stocks at first subculture, retaining one culture in antibiotics as a precaution if necessary. Antibiotics can lead to lax aseptic technique, can inhibit some eukaryotic cellular processes, and can hide the presence of a microbial contamination. If a culture is contaminated this must become apparent as soon as possible, either to indicate that the culture should be discarded before it can spread the contamination to other cultures or to indicate that decontamination should be attempted. The latter should only be used as a last resort; decontamination is not always successful and can lead to the development of antibiotic-resistant organisms.

Most bacterial, fungal, and yeast infections are readily detected by regular careful examination with the naked eye (e.g., by a change in the color of culture medium) and on the microscope. However, one of the most serious contaminations is mycoplasma, which is not visible by routine microscopy. Any cell culture laboratory should have a mycoplasma screening program in operation, but those collecting tissue for primary culture are particularly at risk. The precautions that should be observed are as follows:

- (i) Treat any new material entering the laboratory from donors or from other laboratories as potentially infected and keep it in quarantine. Ideally, a separate room should be set aside for receiving samples and imported cultures. If this is not practicable, handle separately from other cultures, preferably last in the day and in a designated hood, and swab the hood down after use with 2% phenolic disinfectant in 70% alcohol. Use a separate incubator, and adhere strictly to the rules given above regarding medium sharing.
- (ii) Screen new cultures as they arrive, and existing stocks at regular intervals, e.g., once a month. There are a number of tests available, but the most reliable and sensitive are fluorescence microscopy after staining with Hoechst 33258 [Chen et al., 1977; Protocol 19.2, Freshney, 2005] or PCR with a pan-mycoplasma primer [Uphoff and Drexler, 2002a; Protocol 19.3, Freshney, 2005]. The latter is more sensitive but depends on the availability of PCR technology, whereas the former is easier, will detect any DNA-containing contamination, but requires a fluorescence microscope. Both techniques are best performed with so-called *indicator cultures*. The test culture is refreshed with antibiotic-free medium and, after 3–5 days, the medium is transferred to an antibiotic-free, 10% confluent indicator culture of a cell line such as 3T6 or A549 cells, which are well spread and known to support mycoplasma growth. After a further 3 days (the indicator cells must not be allowed to reach confluence) the indicator culture is fixed in acetic methanol and stained with Hoechst 33258, or harvested by scraping for PCR (trypsinization may remove the mycoplasma from the cell surface).
- (iii) Discard all contaminated cultures. If the culture is irreplaceable, decontamination may be attempted (under strict quarantine conditions) with agents

such as Mycoplasma Removal Agent (MRA), ciprofloxacin, or BM-cycline [Uphoff and Drexler, 2002b]. Briefly, the culture is rinsed thoroughly, trypsinized (wash by centrifuging three times after trypsinization), and subcultured into antibiotic-containing medium. This procedure should be repeated for three subcultures and then the culture should be grown up antibiotic-free and tested after one, two, and four further antibiotic-free subcultures, whereupon the culture reenters the routine mycoplasma screening program.

6.3. Characterization

Most laboratories will have, as an integral part of the research program, procedures in place for the characterization of new cultures. Species identification (in case the cells are misidentified or cross-contaminated) will be unnecessary if DNA profiling is being used to confirm cell line identity; otherwise, chromosome analysis or isoenzyme electrophoresis [Hay et al., 2000] can be used. The lineage or tissue or origin can be determined by using antibodies to intermediate filament proteins, for example, cytokeratins for epithelial cells, vimentin for mesodermal cells such as fibroblasts, endothelium, and myoblasts, desmin for myocytes, neurofilament protein for neuronal and some neuroendocrine cells, and glial fibrillary acidic protein for astrocytes. Some cell surface markers are also lineage specific, for example, EMA in epithelial cells, A2B5 in glial cells, PECAM-1 in endothelial cells, and N-CAM in neural cells, and have the additional advantage that they can be used in cell sorting by magnetic sorting or flow cytometry. Morphology can also be used, but can be ambivalent as similarities can exist between cells of very different origins.

Spontaneous transformation is unlikely in normal cells of human origin, but indicators are a more refractile appearance under phase contrast with a lower cytoplasmic/nuclear ratio, piling up of the cells and loss of contact inhibition and density limitation of growth, increased clonogenicity in agar, and the ability to form tumors in immune-deprived hosts, such as the Nude or SCID mouse. Where transformation is detected, it is more likely to be due to cross-contamination, although it is possible that the tissue sample may have contained some preneoplastic cells that have then progressed in culture.

6.4. Differentiation

As stated above, a prerequisite for sustained growth in culture is the ability for the cells to proliferate, and this may preclude differentiation. If differentiation is required, then it is generally necessary for the cells to withdraw from the cell cycle. This can be achieved by removing, or changing, the growth factor supplementation; for example, the O2A common precursor of astrocytes and oligodendrocytes remains as a proliferating precursor cell in PDGF and bFGF, whereas combining bFGF with ciliary neurotropic factor (CNTF) results in differentiation into a type 2 astrocyte [Raff, 1990], and embryonal stem cells, which remain as proliferating primitive cells in the presence of leukemia inhibitory factor (LIF), will

differentiate in the absence of LIF and in the presence of a positively acting factor such as phorbol myristate acetate (PMA, also known as TPA) [Rizzino, 2002].

There are four main parameters governing the entry of cells into differentiation:

- (i) Soluble factors such as growth factors (e.g., EGF, KGF, TGF- β and HGF, NGF), cytokines (IL-6, oncostatin-M, GM-CSF, interferons), vitamins (e.g., retinoids, vitamin D₃, and vitamin K) and calcium [Table 16.1, Freshney, 2000], and planar polar compounds (e.g., DMSO and NaBt) [Tables 17.1, 17.2, Freshney, 2005].
- (ii) Interaction with matrix constituents such as collagen IV, laminin, and proteoglycans. Heparan sulfate proteoglycans (HSPGs), in particular, have a significant role not only in binding to cell surface receptors but also in binding and translocating growth factors and cytokines to high-affinity cell surface receptors [Lopez-Casillas et al., 1993; Filla, 1998].
- (iii) Enhanced cell-cell interaction will also promote differentiation. Homotypic contact interactions can act via gap junctions, which tend to coordinate the response among many like cells in a population by allowing free intercellular flow of second messenger molecules such as cyclic adenosine monophosphate (cAMP) and via cell adhesion molecules such as E-cadherin or N-CAM, which signal via anchorage to the cytoskeleton [Juliano, 2002]. Heterotypic interactions, in solid tissues at least, will tend to act across a basal lamina and are less likely to involve direct cell-cell contact. Signaling is achieved by, on the one hand, modification of the matrix by the mutual contribution of both cell types, and, on the other, by reciprocal transmission of cytokines and growth factors across the basal lamina, such as the transfer of KGF and GM-CSF from dermal fibroblasts to the basal layer of the epidermis in response to IL-1 α and - β diffusing from the epidermis to the dermal fibroblasts [Maas-Szabowski et al., 2002].
- (iv) The position, shape, and polarity of the cells may induce, or at least make the cells permissive for the induction of, differentiation. Epidermal keratinocytes [Maas-Szabowski et al., 2002] and bronchial epithelial cells [Petra et al., 1993] require to be close to the air/liquid interface, presumably to enhance oxygen availability, and secretory cells, such as thyroid epithelium, need the equivalent of the acinar space, that is, no direct access to nutrient or hormones, above them in a thin fluid space [Chambard et al., 1983, 1987]. When cells are grown on collagen at this location, and the collagen gel is allowed to retract, a shape change can occur, for example, from a flat squamous or cuboidal cell into a more columnar morphology, and this, combined with matrix interaction, allows the establishment of polarity in the cells, such that secretory products are released apically and signaling receptors and nutrient transporters locate basally.

Combining these effects *in vitro* may require strict attention to culture geometry, for example, by growing cells in a filter well insert on a matrix incorporating

stromal fibroblasts, and providing differentiation inducers basally in a defined, nonmitogenic medium. Similar conditions may be created in a perfused capillary bed or scaffold.

SOURCES OF MATERIALS

Suppliers of specific materials are listed at the end of each chapter, but the following list provides information on general tissue culture suppliers [See Freshney, 2000 for more detailed list].

<i>Item</i>	<i>Supplier</i>
Antibodies	R & D Systems; Dako; Upstate Biotechnology
BM-cycline	Roche
CellFlo	Spectrum Laboratories
Ciprofloxacin	Bayer
CO ₂ automatic switch-over device	Air Products and Chemicals; Gow-Mac; Lab-Impex; Shandon
CO ₂ incubators	Hereaus; IEC (Hotpak); Lab-Line; Napco; NuAire; Precision Scientific; Thermo-Forma
Collagenase	Sigma; Worthington
Dispase	Roche
DNA profiling or fingerprinting	ATCC; Cellmark Diagnostics; ECACC; Laboratory of the Government Chemist
Electronic cell counter	Beckman-Coulter; Schärfe Systems
FACS	BD Biosciences; Beckman-Coulter
Filter well inserts	BD Biosciences; Corning; Millipore
Growth factors	BD Biosciences; Cambridge Biosciences; GIBCO; ICN; Roche; Sigma; Upstate Biotechnology
Heparan sulfate proteoglycans (HSPGs)	Sigma
Hydrocortisone	Sigma
Inverted microscope	Leica; Olympus; Nikon; Zeiss
Laminar flow hoods (Class II biosafety cabinets)	Atlas Clean Air; Baker; Heto-Holten; Medical Air Technology; NuAire
Liquid nitrogen freezer	Aire Liquide; Cryo-Med; Integra Biosciences; MVE; Taylor Wharton; Thermolyne
MACS	Miltenyi Biotec
Matrix products: heparan sulfate, fibronectin, laminin, collagen, Matrigel	BD Biosciences; Biofluids; Pierce; R & D Systems; Sigma; TCS
Membroferm	Polymun
Mycoplasma Removal Agent (MRA)	ICN
Retinoids	Sigma
Sample containers for transportation (See also www.uos.harvard.edu/ehs/bio_bio_shi.shtml and www.ehs.ucsf.edu/Safety%20Updates/Bsu/Bsu5.pdf)	Saf-T-Pak: 1-800-814-7484; Nalge: 1-716-586-8800; Air Sea Atlanta: 1-404-351-8600; Cellutech: 1-800-575-5945; Cin-Made Corp: 1-513-681-3600
Serum-free media	Cambrex; PromoCell; Cascade Biologicals; Sigma; GIBCO
Sodium butyrate (NaBt)	Sigma
Trypsin	Cambrex; GIBCO; ICN; Sigma; Worthington

REFERENCES

- Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C.-P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S., Wright, W.E. (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* 279: 349–352.
- Caputo, J.L. (1996) Safety Procedures. In Freshney, R.I., Freshney, M.G., eds., *Culture of Immortalized Cells*. New York, Wiley-Liss, pp. 25–51.
- Carr, T., Evans, P., Campbell, S., Bass, P., Albano, J. (1999) Culture of human renal tubular cells: positive selection of kallikrein-containing cells. *Immunopharmacology* 44: 161–167.
- Cerni, C. (2000) Telomeres, telomerase, and myc. An update. *Mutat Res.* 462: 31–47.
- Chambard, M., Vemer, B., Gabrion, J., Mauchamp, J., Bugeia, J.C., Pelassy, C., Mercier, B. (1983) Polarization of thyroid cells in culture; evidence for the basolateral localization of the iodide “pump” and of the thyroid-stimulating hormone receptor-adenyl cyclase complex. *J. Cell Biol.* 96: 1172–1177.
- Chambard, M., Mauchamp, J., Chaband, O. (1987) Synthesis and apical and basolateral secretion of thyroglobulin by thyroid cell monolayers on permeable substrate: Modulation by thyrotropin. *J. Cell Physiol.* 133: 37–45.
- Chen, T.R. (1977) In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp. Cell Res.* 104: 255.
- Filla, M.S., Dam, P., Rapraeger, A.C. (1998) The cell surface proteoglycan syndecan-1 mediates fibroblast growth factor-2 binding and activity. *J. Cell Physiol.* 174: 310–321.
- Freshney, R.I. (2002) Cell line provenance. *Cytotechnology* 39: 3–15.
- Freshney, R.I. (2005) *Culture of Animal Cells, a Manual of Basic Technique*, 5th Ed. Hoboken NJ, John Wiley & Sons.
- Greco, B., Recht, L. (2003) Somatic plasticity of neural stem cells: Fact or fancy? *J. Cell. Biochem.* 88: 51–56.
- Ham, R.G., McKeehan, W.L. (1978) Development of improved media and culture conditions for clonal growth of normal diploid cells. *In Vitro* 14: 11–22.
- Hay, R.J., Miranda-Cleland, M., Durkin, S., Reid, Y.A. (2000) Cell line preservation and authentication. In Masters, J.R.W, ed., *Animal Cell Culture*, 3rd Ed. Oxford, Oxford University Press, pp. 69–103.
- Juliano, R.L. (2002) Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. *Annu. Rev. Pharmacol. Toxicol.* 42: 283–323.
- Karmiol, S. (2000) Development of serum-free media. In Masters, J.R.W, ed., *Animal Cell Culture*, 3rd Ed. Oxford, Oxford University Press, pp. 105–121.
- Klement, G., Scheirer, W., Katinger, H.W. (1987) Construction of a large scale membrane reactor system with different compartments for cells, medium and product. *Dev Biol Stand.*, 66: 221–226.
- Knazek, R.A., Gullino, P., Kohler, P.O., Dedrick, R. (1972) Cell culture on artificial capillaries. An approach to tissue growth in vitro. *Science* 178: 65–67.
- Kruse, P.F., Jr., Keen, L.N., Whittle, W.L. (1970) Some distinctive characteristics of high density perfusion cultures of diverse cell types. *In Vitro* 6: 75–78.
- Laning, J.C., DeLuca, J.E., Hardin-Young, J. (1999) Effects of immunoregulatory cytokines on the immunogenic potential of the cellular components of a bilayered living skin equivalent. *Tissue Eng.* 5: 171–181.
- Lopez-Casillas, F., Wrana, J.L., Massague, J. (1993) Betaglycan presents ligand to the TGF- β signalling receptor. *Cell* 73: 1435–1444.
- Maas-Szabowski, N., Stark, H.-J., Fusenig, N.E. (2002) Cell interaction and differentiation. In Freshney, R.I. and Freshney, M.G., eds, *Culture of Epithelial Cells*, 2nd Ed. New York, John Wiley & Sons, pp. 31–63.
- MacLeod, R.A., Dirks, W.G., Matsuo, Y., Kaufmann, M., Milch, H., Drexler, H.G. (1999) Widespread intraspecies cross-contamination of human tumor cell lines arising at source. *Int. J. Cancer* 83: 555–63.
- Marcovic, O., Marcovic, N. (1998) Cell cross-contamination in cell cultures: the silent and neglected danger. *In Vitro Cell Dev Biol.* 34: 108.

- Masters, J.R. (2002) HeLa cells 50 years on: the good, the bad and the ugly. *Nat. Rev. Cancer* 2: 315–9.
- Masters, J.R.W., Thomson, J.A., Daly-Burns, B., Reid, Y.A., Dirks, W.G., Packer, P., Toji, L.H., Ohno, T., Tanabe, H., Arlett, C.F., Kelland, L.R., Harrison, M., Virmani, A., Ward, T.H., Ayres, K.L., Debenham, P.G. (2001) STR profiling provides an international reference standard for human cell lines. *Proc. Natl. Acad. Sci. USA* 98: 8012–8017.
- Mather, J.P. (1998) Making informed choices: medium, serum, and serum-free medium. How to choose the appropriate medium and culture system for the model you wish to create. *Methods Cell Biol.* 57: 19–30.
- Michel, M., L’Heureux, N., Pouliot, R., Xu, W., Auger, F.A., Lucie, G. (1999) Characterization of a new tissue-engineered human skin equivalent with hair. *In Vitro Cell. Dev. Biol. Anim.* 35: 318–326.
- Munger, K., Howley, P.M. (2002) Human papillomavirus immortalization and transformation functions. *Virus Res.* 89(2): 213–228.
- Peehl, D.M., Ham, R.G. (1980) Clonal growth of human keratinocytes with small amounts of dialysed serum. *In Vitro* 16: 526–540.
- Petra, M., de Jong, P.M., van Sterkenburg, A.J.A., Kempenaar, J.A., Dijkman, J.H., Ponc, M. (1993) Serial culturing of human bronchial epithelial cells derived from biopsies. *In Vitro Cell Dev. Biol.* 29A: 379–387.
- Pretlow, T.G., and Pretlow, T.P. (1989) Cell separation by gradient centrifugation methods. *Methods Enzymol.* 171: 462–482.
- Raff, M.C. (1990) Glial cell diversification in the rat optic nerve. *Science* 243: 1450–1455.
- Regnier, M., Staquet, M.J., Schmitt, D., Schmidt, R. (1997) Integration of Langerhans cells into a pigmented reconstructed human epidermis. *J. Invest. Dermatol.* 109: 510–512.
- Rizzino, A. (2002) Embryonic stem cells provide a powerful and versatile model system. *Vitam Horm.* 64: 1–42.
- Saalbach, A., Aust, G., Hausteiner, U.F., Herrmann, K., Anderegg, U. (1997) The fibroblast-specific MAb AS02: A novel tool for detection and elimination of human fibroblasts. *Cell Tissue Res.* 290: 593–599.
- Schaller, M., Mailhammer, R., Grassl, G., Sander, C.A., Hube, B., Korting, H.C. (2002) Infection of human oral epithelia with *Candida* species induces cytokine expression correlated to the degree of virulence. *J. Invest. Dermatol.* 118: 652–657.
- Stoker, M.G.P. (1973) Role of diffusion boundary layer in contact inhibition of growth. *Nature* 246: 200–203.
- Swope, V.B., Supp, A.P., Cornelius, J.R., Babcock, G.F., Boyce, S.T. (1997) Regulation of pigmentation in cultured skin substitutes by cytometric sorting of melanocytes and keratinocytes. *J. Invest. Dermatol.* 109: 289–295.
- Uphoff, C.C., Drexler, H.G. (2002a) Comparative PCR analysis for detection of mycoplasma infection in continuous cell lines. *In Vitro Cell. Dev. Biol. Anim.* 38: 79–85.
- Uphoff, C.C., and Drexler, H.G. (2002b) Comparative antibiotic eradication of mycoplasma infections from continuous cell lines. *In Vitro Cell. Dev. Biol. Anim.* 38: 86–89.
- van Bokhoven, A., Varella-Garcia, M., Korch, C., Hessels, D., Miller, G.J. (2001). Widely used prostate carcinoma cell lines share common origins. *Prostate* 47: 36–51.
- Westermarck, B., Wasteson, A. (1975) The response of cultured human normal glial cells to growth factors. *Adv. Metab. Disord.* 8: 85–100.
- Wright, W.E., Shay, J.W. (2002) Historical claims and current interpretations of replicative aging. *Nat. Biotechnol.* 20: 682–688.