CHAPTER 3

Biology of Cultured Cells

3.1 THE CULTURE ENVIRONMENT

The validity of the cultured cell as a model of physiological function *in vivo* has frequently been criticized. Often, the cell does not express the correct *in vivo* phenotype because the cell's microenvironment has changed. Cell–cell and cell–matrix interactions are reduced because the cells lack the heterogeneity and three-dimensional architecture found *in vivo*, and many hormonal and nutritional stimuli are absent. This creates an environment that favors the spreading, migration, and proliferation of unspecialized progenitor cells, rather than the expression of differentiated functions. The influence of the environment on the culture is expressed via four routes: (1) the nature of the substrate on or in which the cells grow—solid, as on plastic or other rigid matrix, semisolid, as in a gel such as collagen or agar, or liquid, as in a suspension culture; (2) the degree of contact with other cells; (3) the physicochemical and physiological constitution of the medium; (4) the constitution of the gas phase; and (5) the incubation temperature. The provision of the appropriate environment, including substrate adhesion, nutrient and hormone or growth factor concentration, and cell interaction, is fundamental to the expression of specialized functions (*see* Sections 17.1, 17.7 and Alberts et al., 2002).

3.2 CELL ADHESION

Most cells from solid tissues grow as adherent monolayers, and, unless they have transformed and become anchorage independent (*see* Section 18.5.1), after tissue disaggregation or subculture they will need to attach and spread out on the substrate before they will start to proliferate (*see* Sections 13.7, 21.9.2). Originally, it was found that cells would attach to, and spread on, glass that had a slight net negative charge. Subsequently, it was shown that cells would attach to some plastics, such as polystyrene, if the plastic was appropriately treated with an electric ion discharge or high-energy ionizing radiation. We now know that cell adhesion is mediated by specific cell surface receptors for molecules in the extracellular matrix (*see also* Sections 8.4, 17.7.3), so it seems likely that spreading may be preceded by the secretion of extracellular matrix proteins and proteoglycans by the cells. The matrix adheres to the charged substrate, and the cells then bind to the matrix via specific receptors. Hence, glass or plastic that has been conditioned by previous cell growth can often provide a better surface for attachment, and substrates pretreated with matrix constituents, such as fibronectin or collagen, or derivatives, such as gelatin, will help the more fastidious cells to attach and proliferate.

With fibroblast-like cells, the main requirement is for substrate attachment and spreading and the cells migrate individually at low densities. Epithelial cells may also require cell–cell adhesion for optimum survival and growth and, consequently, they tend to grow in patches.

3.2.1 Cell Adhesion Molecules

Three major classes of transmembrane proteins have been shown to be involved in cell–cell and cell–substrate adhesion (Fig. 3.1). Cell–cell adhesion molecules, CAMs (Ca^{2+} independent), and *cadherins* (Ca^{2+} dependent) are involved primarily in interactions between homologous cells. These

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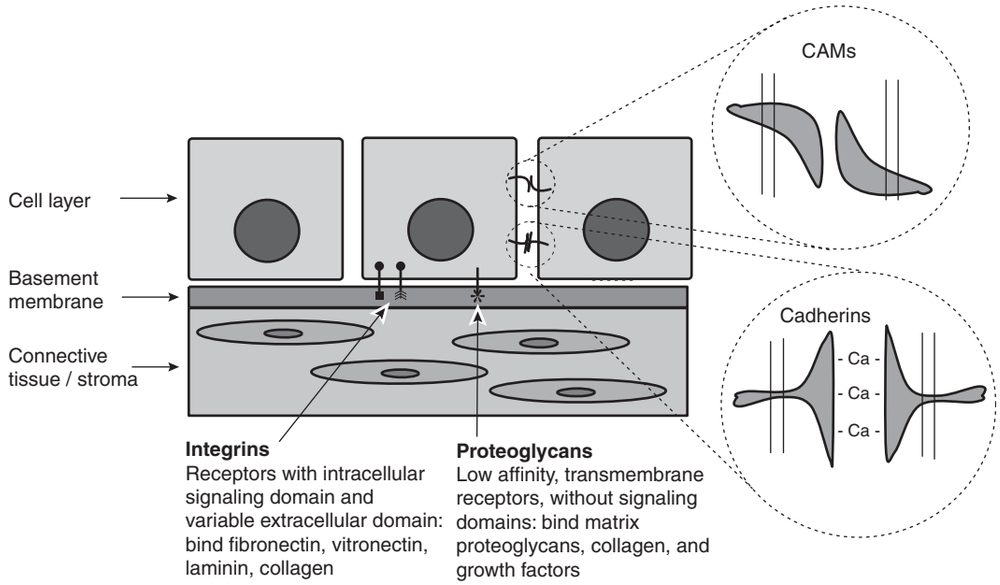

Fig. 3.1. Cell Adhesion. Diagrammatic representation of a layer of epithelial cells above connective tissue containing fibrocytes and separated from it by a basal lamina. CAMs and cadherins are depicted between like cells, integrins and proteoglycans between the epithelial layer and the matrix of the basal lamina.

proteins are self-interactive; that is, homologous molecules in opposing cells interact with each other [Rosenman & Gallatin, 1991; Alberts et al., 2002], and the cell–cell recognition that this generates has a signaling role in cell behavior [Cavallaro & Christofori, 2004]. Cell–substrate interactions are mediated primarily by *integrins*, receptors for matrix molecules such as fibronectin, entactin, laminin, and collagen, which bind to them via a specific motif usually containing the arginine–glycine–aspartic acid (RGD) sequence [Yamada & Geiger, 1997]. Each integrin comprises one α and one β subunit, the extracellular domains of which are highly polymorphic, thus generating considerable diversity among the integrins. Both integrins and cadherins interact with vinculin, a step in signaling to the nucleus [Bakolitsa et al., 2004].

The third group of cell adhesion molecules is the transmembrane proteoglycans, also interacting with matrix constituents such as other proteoglycans or collagen, but not via the RGD motif. Some transmembrane and soluble proteoglycans also act as low-affinity growth factor receptors [Subramanian et al., 1997; Yevdokimova & Freshney, 1997] and may stabilize, activate, and/or translocate the growth factor to the high-affinity receptor, participating in its dimerization [Schlessinger et al., 1995].

Disaggregation of the tissue, or an attached monolayer culture, with protease will digest some of the extracellular matrix and may even degrade some of the extracellular domains of transmembrane proteins, allowing cells to become dissociated from each other. Epithelial cells are generally more resistant to disaggregation, as they tend to have tighter junctional complexes (desmosomes, adherens junctions, and tight junctions) holding them together, whereas mesenchymal cells, which are more dependent on matrix interactions for intercellular bonding, are more easily dissociated. Endothelial cells may also express tight junctions in culture, especially if left at confluence for prolonged periods on a preformed matrix, and can be difficult to dissociate. In each case, the cells must resynthesize matrix proteins before they attach or must be provided with a matrix-coated substrate.

3.2.2 Intercellular Junctions

Although some cell adhesion molecules are diffusely arranged in the plasma membrane, others are organized into intercellular junctions. The role of the junctions varies between mechanical, such as the desmosomes and adherens junctions, which hold epithelial cells together, tight junctions, which seal the space between cells, e.g. between secretory cells in an acinus or duct or between endothelial cells in a blood vessel, and gap junctions, which allow ions, nutrients, and small signaling molecules such as cyclic adenosine monophosphate (cAMP) to pass between cells in contact [*see* Alberts et al., 2002]. Although desmosomes may be distributed throughout the area of plasma membranes in contact (Fig. 3.2a), they are often associated with tight and adherens junctions at the apical end of lateral cell contacts (Fig. 3.2b).

As epithelial cells differentiate in confluent cultures they can form an increasing number of desmosomes and, if

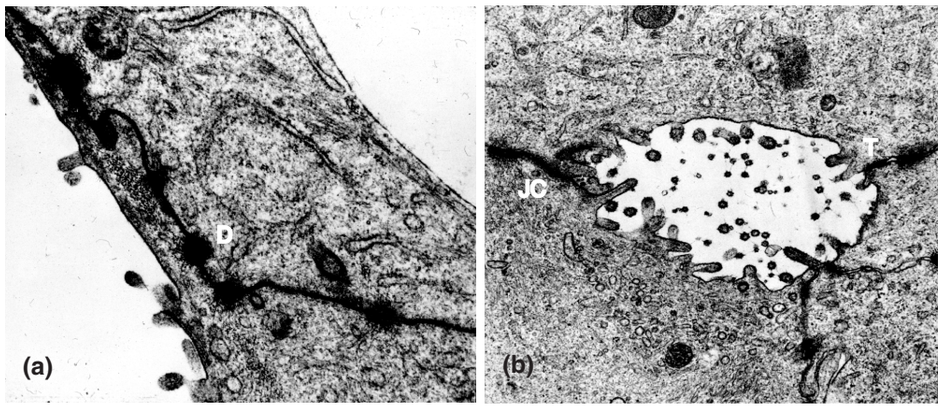

Fig. 3.2. Intercellular Junctions. Electron micrograph of culture of CA-KD cells, an early-passage culture from an adenocarcinoma secondary in brain (primary site unknown). Cells grown on Petriperm dish (Vivascience). (a) Desmosomes (D) between two cells in contact; mag. 28,000×. (b) Canaliculus showing tight junctions (T) and junctional complex (JC); mag. 18,500×. (Courtesy of Carolyn MacDonald).

some morphological organization occurs, can form complete junctional complexes. This is one reason why epithelial cells, if left at confluence for too long, can be difficult to disaggregate. As many of the adhesion molecules within these junctions depend on Ca^{2+} ions, a chelating agent, such as EDTA, is often added to the trypsin during or before disaggregation.

3.2.3 Extracellular Matrix

Intercellular spaces in tissues are filled with extracellular matrix (ECM), the constitution of which is determined by the cell type, e.g., fibrocytes secrete type I collagen and fibronectin into the matrix, whereas epithelial cells produce laminin. Where adjacent cell types are different, e.g., at the boundary of the dermis (fibrocytes) and epidermis (epithelial keratinocytes), both cell types will contribute to the composition of the ECM, often producing a *basal lamina*. The complexity of the ECM is a significant component in the phenotypic expression of the cells attached to it, so a dynamic equilibrium exists in which the cells attached to the ECM control its composition and, in turn, the composition of the ECM regulates the cell phenotype [Kleinman et al., 2003; Zoubiane et al., 2003; Fata et al., 2004]. Hence a proliferating, migratory fibroblast will require a different ECM from a differentiating epithelial cell or neuron. Mostly, cultured cell lines are allowed to generate their own ECM, but primary culture and propagation of some specialized cells, and the induction of their differentiation, may require exogenous provision of ECM.

ECM is comprised variously of collagen, laminin, fibronectin, hyaluronan, proteoglycans, and bound growth factors or cytokines [Alberts et al., 1997, 2002]. It can be prepared by mixing purified constituents, such as collagen and fibronectin, by using cells to generate ECM and washing the producer cells off before reseeding with the cells under study (*see* Protocol 8.1), or by using a preformed matrix generated by the Engelberth-Holm-Swarm (EHS) mouse sarcoma, available commercially as Matrigel (*see* Section 8.4.1). Matrigel is often used to encourage differentiation and morphogenesis in culture and frequently generates a latticelike network with epithelial (Fig 3.3; Plate 12c) or endothelial cells.

At least two components of interaction with the substrate may be recognized: (1) adhesion, to allow the attachment and spreading that are necessary for cell proliferation [Folkman & Moscona, 1978], and (2) specific interactions, reminiscent of the interaction of an epithelial cell with basement membrane, with other ECM constituents, or with adjacent tissue cells, and required for the expression of some specialized functions (*see* Sections 3.4.1 and 17.7.3). Rojkind et al. [1980], Vlodavsky et al. [1980], and others explored the growth of cells on other natural substrates related to basement membrane. Natural matrices and defined-matrix macromolecules such as Matrigel, Natrigel, collagen, laminin, and vitronectin (B-D Biosciences, Invitrogen) are now available for controlled studies on matrix interaction.

The use of ECM constituents can be highly beneficial in enhancing cell survival, proliferation, or differentiation, but, unless recombinant molecules are used [*see*, e.g., Ido et al., 2004] there is a significant risk of the introduction of adventitious agents from the originating animal (*see* Section 10.1). Fibronectin and laminin fragments are now available commercially (*see* Appendix II).

3.2.4 Cytoskeleton

Cell adhesion molecules are attached to elements of the cytoskeleton. The attachment of integrins to actin microfilaments via linker proteins is associated with reciprocal signaling between the cell surface and the nucleus [Fata et al., 2004]. Cadherins can also link to the actin cytoskeleton

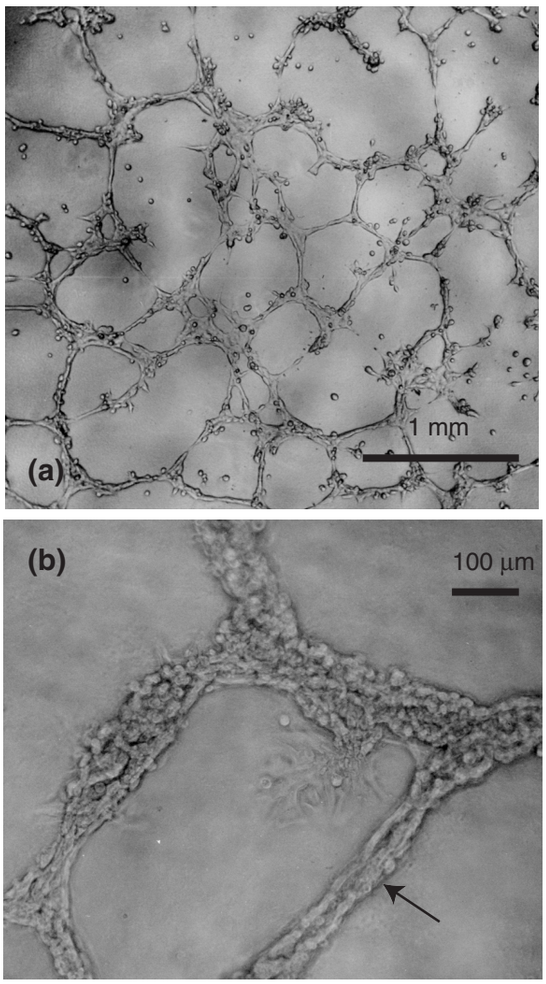

Fig. 3.3. A549 Cells Growing on Matrigel. Cultures of A549 adenocarcinoma cells growing on Matrigel. (a) Low-power shot showing lattice formation 24 h after seeding at 1×10^5 cells/mL. (b) Higher power, 3 days after seeding at 1×10^5 cells/mL. Arrow indicates possible tubular formation. (Courtesy of Jane Sinclair; *see also* Plate 12c.)

in adherens junctions, mediating changes in cell shape. Desmosomes, which also employ cadherins, link to the intermediate filaments—in this case, cytokeratins—via an intracellular plaque, but it is not yet clear whether this linkage is a purely structural feature or also has a signaling capacity. Intermediate filaments are specific to cell lineages and can be used to characterize them (*see* Section 16.3.2; Plate 11a–c). The microtubules are the third component of the cytoskeleton; their role appears to be related mainly to cell motility and intracellular trafficking of micro-organelles, such as the mitochondria and the chromatids at cell division.

3.2.5 Cell Motility

Time-lapse recording (*see* Section 27.3) demonstrates that cultured cells are capable of movement on a substrate. The most motile are fibroblasts at a low cell density (when cells are not in contact), and the least motile are dense epithelial monolayers. Fibroblasts migrate as individual cells with a recognizable polarity of movement. A lamellipodium, generated by polymerization of actin [Pollard & Borisy, 2003], extends in the direction of travel and adheres to the substrate, and the plasma membrane at the opposite side of the cell retracts, causing the cell to undergo directional movement. If the cell encounters another cell, the polarity reverses, and migration proceeds in the opposite direction. Migration proceeds in erratic tracks, as revealed by colloidal gold tracking [Scott et al., 2000], until the cell density reaches confluence, whereupon directional migration ceases. The cessation of movement at confluence, which is accompanied by a reduction in plasma membrane ruffling, is known as *contact inhibition* (*see* Section 18.5.2) and leads eventually to withdrawal of the cell from the division cycle. Myoblasts and endothelial cells migrate in a similar fashion and, like fibroblasts, may differentiate when they reach confluence, depending on the microenvironment.

Epithelial cells, unless transformed, tend not to display random migration as polarized single cells. When seeded at a low density, they will migrate until they make contact with another cell and the migration stops. Eventually, cells accumulate in patches and the whole patch may show signs of coordinated movement [Casanova, 2002].

3.3 CELL PROLIFERATION

3.3.1 Cell Cycle

The cell cycle is made up of four phases (Fig. 3.4). In the *M phase* (M = mitosis), the chromatin condenses into chromosomes, and the two individual chromatids, which make up the chromosome, segregate to each daughter cell. In the G_1 (Gap 1) *phase*, the cell either progresses toward DNA

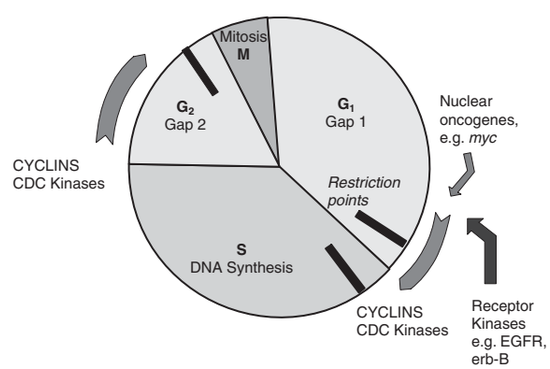

Fig. 3.4. Cell Cycle. The cell cycle is divided into four phases: G_1, S, G_2, and M. Progression round the cycle is driven by cyclins interacting with CDC kinases and stimulated by nuclear oncogenes and cytoplasmic signals initiated by receptor kinase interaction with ligand. The cell cycle is arrested at restriction points by cell cycle inhibitors such as Rb and p53.

synthesis and another division cycle or exits the cell cycle reversibly (G_0) or irreversibly to commit to differentiation. It is during G_1 that the cell is particularly susceptible to control of cell cycle progression at a number of restriction points, which determine whether the cell will re-enter the cycle, withdraw from it, or withdraw and differentiate. G_1 is followed by the *S phase* (DNA synthesis), in which the DNA replicates. S in turn is followed by the G_2 (Gap 2) *phase* in which the cell prepares for reentry into mitosis. Checkpoints at the beginning of DNA synthesis and in G_2 determine the integrity of the DNA and will halt the cell cycle to allow DNA repair or entry into apoptosis if repair is impossible. Apoptosis, or programmed cell death [al-Rubeai & Singh, 1998], is a regulated physiological process whereby a cell can be removed from a population. Marked by DNA fragmentation, nuclear blebbing, and cell shrinkage (*see* Plate 17c,d), apoptosis can also be detected by a number of marker enzymes with kits such as Apotag (Oncor) or the COMET assay [Maskell & Green, 1995].

3.3.2 Control of Cell Proliferation

Entry into the cell cycle is regulated by signals from the environment. Low cell density leaves cells with free edges and renders them capable of spreading, which permits their entry into the cycle in the presence of mitogenic growth factors, such as epidermal growth factor (EGF), fibroblast growth factors (FGFs), or platelet-derived growth factor (PDGF) (*see* Sections 9.5.2, 10.4.3 and Table 10.3), interacting with cell surface receptors. High cell density inhibits the proliferation of normal cells (though not transformed cells) (*see* Section 18.5.2). Inhibition of proliferation is initiated by cell contact and is accentuated by crowding and the resultant change in the shape of the cell and reduced spreading.

Intracellular control is mediated by positive-acting factors, such as the cyclins [Planas-Silva & Weinberg, 1997; Reed, 2003] (*see* Fig. 3.2), which are upregulated by signal transduction cascades activated by phosphorylation of the intracellular domain of the receptor when it is bound to growth factor. Negative-acting factors such as p53 [Sager, 1992; McIlwrath et al., 1994], p16 [Russo et al., 1998], or the Rb gene product [Sager, 1992] block cell cycle progression at restriction points or checkpoints (Fig. 3.5). The link between the extracellular control elements (both positive-acting, e.g., PDGF, and negative-acting, e.g., TGF-β) and intracellular effectors is made by cell membrane receptors and signal transduction pathways, often involving protein phosphorylation and second messengers such as cAMP, Ca^{2+}, and diacylglycerol [Alberts et al., 2002]. Much of the evidence for the existence of these steps in the control of cell proliferation has emerged from studies of oncogene and suppressor gene expression in tumor cells, with the ultimate objective of the therapeutic regulation of uncontrolled cell proliferation in cancer. The immediate benefit, however, has been a better understanding of the factors required to regulate cell proliferation in culture [Jenkins, 1992]. These studies have had other benefits as well, including the identification of genes that enhance cell proliferation, some of which can be used to immortalize finite cell lines (*see* Section 18.4).

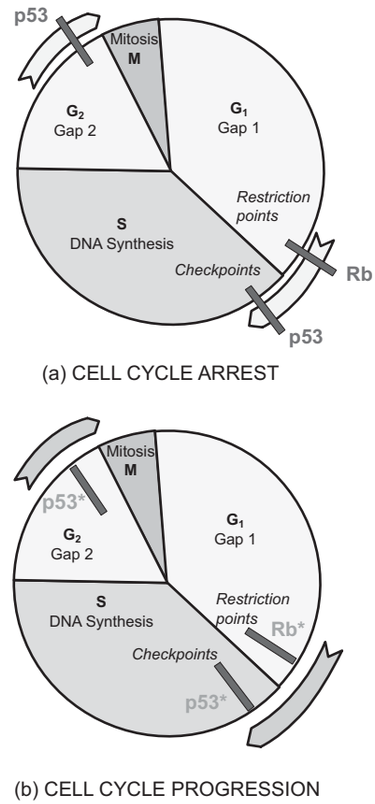

Fig. 3.5. Cell Cycle Inhibition and Progression. The cell cycle is arrested at restriction points or checkpoints by the action of Rb, p53, and other cell cycle inhibitors (a). When these are inactivated, usually by phosphorylation, cells proceed round the cycle (b).

3.4 DIFFERENTIATION

As stated earlier (*see* Section 1.3.3), the expression of differentiated properties in cell culture is often limited by the promotion of cell proliferation, which is necessary for the propagation of the cell line and the expansion of stocks. The conditions required for the induction of differentiation—a high cell density, enhanced cell–cell and cell–matrix interaction, and the presence of various differentiation factors (*see* Sections 17.1.1, 17.7)—may often be antagonistic to cell proliferation and vice versa. So if differentiation is required, it may be necessary to define two distinct sets of conditions—one to optimize cell proliferation and one to optimize cell differentiation.

3.4.1 Maintenance of Differentiation

It has been recognized for many years that specific functions are retained longer when the three-dimensional structure of

the tissue is retained, as in organ culture (*see* Section 25.2). Unfortunately, organ cultures cannot be propagated, must be prepared *de novo* for each experiment, and are more difficult to quantify than cell cultures. Re-creating three-dimensional structures by perfusing monolayer cultures (*see* Sections 25.3, 26.2.6) and culturing cells on or in special matrices, such as collagen gel, cellulose, or gelatin sponge, or other matrices (*see* Sections 3.2.3, 8.4.1, 8.4.3, 17.7.3) may be a better option. A number of commercial products, the best known of which is Matrigel (BD Biosciences), reproduce the characteristics of extracellular matrix, but are undefined, although a growth factor-depleted version is also available (GFR Matrigel). These techniques present some limitations, but with their provision of homotypic cell interactions and cell–matrix interactions, and with the possibility of introducing heterotypic cell interactions, they hold considerable promise for the examination of tissue-specific functions, particularly when interactions may be regulated by growing cultures in filter-well inserts (*see* Section 25.3.6). Expression of the differentiated phenotype may also require maintenance in the appropriate selective medium (*see* Section 10.2.1), with appropriate soluble inducers, such as hydrocortisone, retinoids, or planar polar compounds (*see* Sections 17.7.1, 17.7.2), and usually in the absence of serum.

The development of normal tissue functions in culture would facilitate the investigation of pathological behavior such as demyelination and malignant invasion. However,

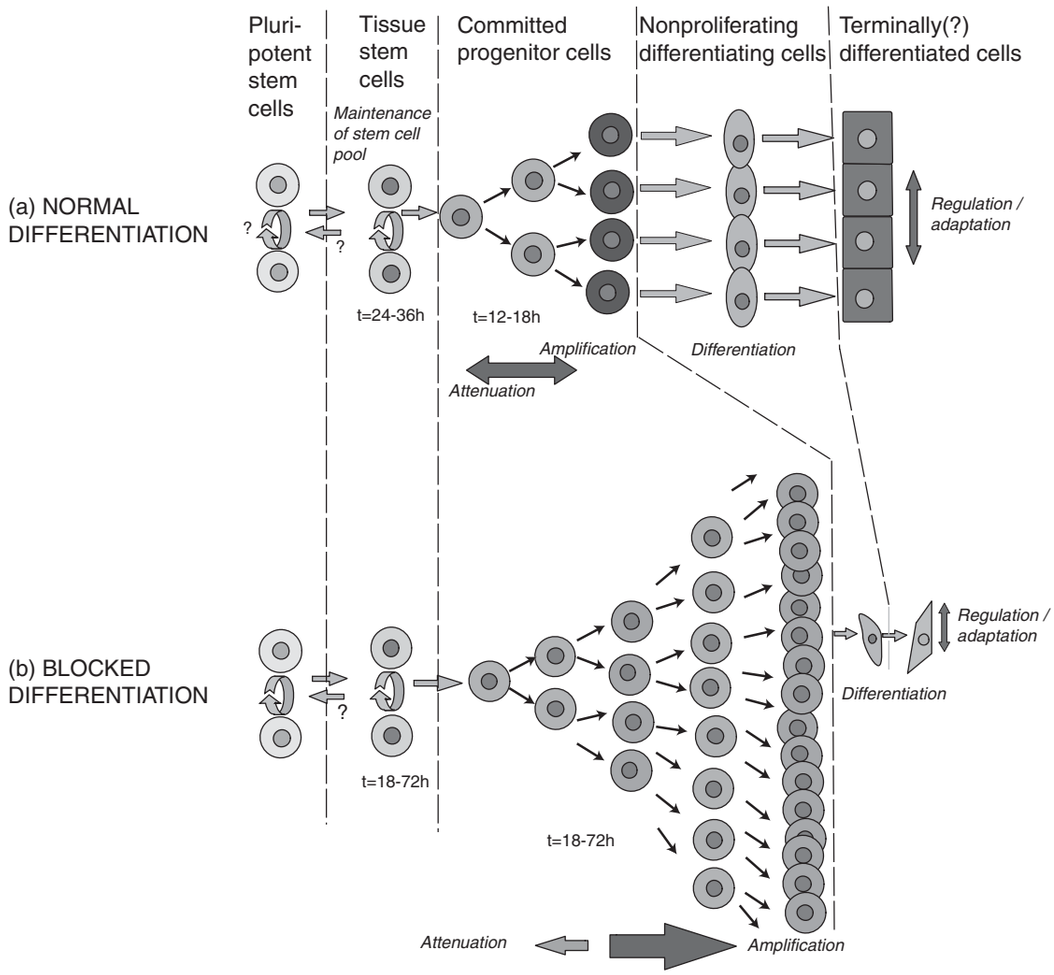

Fig. 3.6. Differentiation from Stem Cells. (a) *In vivo*, a small stem cell pool gives rise to a proliferating progenitor compartment that produces the differentiated cell pool. (b) *In vitro*, differentiation is limited by the need to proliferate, and the population becomes predominantly progenitor cells, although stem cells may also be present. Pluripotent stem cells (far left) have also been cultured from some tissues, but their relationship to the tissue stem cells is as yet unclear. Culture conditions select mainly for the proliferating progenitor cell compartment of the tissue or induce cells that are partially differentiated to revert to a progenitor status.

from a fundamental viewpoint, it is only when cells *in vitro* express their normal functions that any attempt can be made to relate them to their tissue of origin. The expression of the differentiated phenotype need not be complete, because the demonstration of a single type-specific surface antigen may be sufficient to place a cell in the correct lineage. More complete functional expression may be required, however, to place a cell in its correct *position* in the lineage and to reproduce a valid model of its function *in vivo*.

3.4.2 Dedifferentiation

Historically, the inability of cell lines to express the characteristic *in vivo* phenotype was blamed on *dedifferentiation*. According to this concept, differentiated cells lose their specialized properties *in vitro*, but it is often unclear whether (1) the wrong lineage of cells is selected *in vitro*, (2) undifferentiated cells of the same lineage (Fig. 3.6) overgrow terminally differentiated cells of reduced proliferative capacity, or (3) the absence of the appropriate

TABLE 3.1. Cell Lines with Differentiated Properties

Cell type	Origin	Cell line	*N**	Species	Marker	Reference
Endocrine	Adrenal cortex	Y-1	T	Mouse	Adrenal steroids	Yasamura et al., 1966
Endocrine	Pituitary tumor	GH3	T	Rat	Growth hormone	Buonassisi et al., 1962
Endocrine	Hypothalamus	C7	N	Mouse	Neurophysin; vasopressin	De Vitry et al., 1974
Endothelium	Dermis	HDMEC		Human	Factor VIII, CD36	Gupta et al., 1997
Endothelium	Pulmonary artery	CPAE	C	Cow	Factor VIII, angiotensin II-converting enzyme	Del Vecchio & Smith, 1981
Endothelium	Hepatoma	SK/HEP-1	T	Human	Factor VIII	Heffelfinger et al., 1992
Epithelium	Prostate	PPEC	N	Human	PSA	Robertson & Robertson, 1995
Epithelium	Kidney	MDCK	C	Dog	Domes, transport	Gaush et al., 1966; Rindler et al., 1979
Epithelium	Kidney	LLC–PKI	C	Pig	Na^+-dependent glucose uptake	Hull et al., 1976; Saier, 1984
Epithelium	Breast	MCF-7	T	Human	Domes, α-lactalbumin	Soule et al., 1973
Glia	Glioma	MOG–G-CCM	T	Human	Glutamyl synthetase	Balmforth et al., 1986
Glia	Glioma	C6	T	Rat	Glial fibrillary acidic protein, GPDH	Benda et al., 1968
Hepatocytes	Hepatoma	H4–11–E–C3	T	Rat	Tyrosine aminotransferase	Pitot et al., 1964
Hepatocytes	Liver		T	Mouse	Aminotransferase	Yeoh et al., 1990
Keratinocytes	Epidermis	HaCaT	C	Human	Cornification	Boukamp et al., 1988
Leukemia	Spleen	Friend	T	Mouse	Hemoglobin	Scher et al., 1971
Melanocytes	Melanoma	B16	T	Mouse	Melanin	Nilos & Makarski, 1978
Myeloid	Leukemia	K562	T	Human	Hemoglobin	Andersson et al., 1979a, b
Myeloid	Myeloma	Various	T	Mouse	Immunoglobulin	Horibata & Harris, 1970
Myeloid	Marrow	WEHI–3B D+	T	Mouse	Morphology	Nicola, 1987
Myeloid	Leukemia	HL60	T	Human	Phagocytosis; Neotetrazolium Blue reduction	Olsson & Ologsson, 1981
Myocytes	Skeletal muscle	C2	C	Mouse	Myotubes	Morgan et al., 1992
		L6	C	Rat	Myotubes	Richler & Yaffe, 1970
Neuroendocrine	Pheochromocytoma	PC12	T	Rat	Catecholamines; dopamine; norepinephrine	Greene & Tischler, 1976
Neurons	Neuroblastoma	C1300	T	Rat	Neurites	Lieberman & Sachs, 1978
Type II pneumocyte or Clara cell	Lung carcinoma	A549	T	Human	Surfactant	Giard et al., 1972
		NCI-H441	T	Human	Surfactant	Brower et al., 1986
Type II pneumocyte	Lung carcinoma		I	Mouse	Surfactant	Wilkenheiser et al., 1991
Various	Embryonal teratocarcinoma	F9	T	Mouse	PA, laminin, type IV collagen	Bernstine et al., 1973

*Normal (N), continuous (C), immortalized (I), transformed (T).

inducers (hormones: cell or matrix interaction) causes an adaptive, and potentially reversible, loss of differentiated properties (*see* Section 17.1.1). In practice, all of these may contribute to loss of differentiation; even in the correct lineage-selective conditions, continuous proliferation will favor undifferentiated precursors, which, in the absence of the correct inductive environment, do not differentiate.

An important distinction should be made between dedifferentiation, deadaptation, and selection. Dedifferentiation implies that the specialized properties of the cell are lost by conversion to a more primitive phenotype. For example, a hepatocyte would lose its characteristic enzymes (arginase, aminotransferases, etc.) and could not store glycogen or secrete serum proteins, because of reversion or conversion to a precursor cell [Kondo & Raff, 2004]. Deadaptation, on the other hand, implies that the synthesis of specific products or other aspects of specialized function are under regulatory control by hormones, cell–cell interaction, cell–matrix interaction, etc., and can be reinduced if the correct conditions can be re-created. For instance, the presence of matrix as a floating collagen raft [Michalopoulos & Pitot, 1975], Matrigel [Bissell et al., 1987], or dimethyl sulfoxide (DMSO) [Cable & Isom, 1997] allows retention of differentiated properties in hepatocytes. It is now clear that, given the correct culture conditions, differentiated functions can be reexpressed (Table 3.1; *see also* Section 17.5).

For induction to occur, the appropriate cells must be present. In early attempts at liver cell culture, the failure of cells to express hepatocyte properties was due partly to overgrowth of the culture by connective tissue fibroblasts or endothelium from blood vessels or sinusoids. With the correct disaggregation technique and the correct culture conditions [Guguen-Guillouzo, 2002] (*see also* Protocol 23.6), hepatocytes can be selected preferentially. Similarly, epidermal cells can be grown by using either a confluent feeder layer [Rheinwald & Green, 1975] or a selective medium [Peehl & Ham, 1980; Tsao et al., 1982] (*see* Protocol 23.1). Selective media also have been used for many other types of epithelium [Freshney, 2002]. These and other examples [e.g., selective feeder layers (*see* Protocols 23.1, 23.4, 24.1), D-valine for the isolation of kidney epithelium, and the use of cytotoxic antibodies (*see* Section 14.6)] clearly demonstrate that the selective culture of specialized cells is achievable. Many selective media, based mainly on supplemented Ham's F12:DMEM or modifications of the MCDB series (*see* Section 10.2.1), have been devised [Cartwright & Shah, 1994; Mather, 1998], and many are now available commercially (*see* Appendix II), often with specialized cultures.

3.5 CELL SIGNALING

Cell proliferation, migration, differentiation, and apoptosis *in vivo* are regulated by cell–cell interaction, cell–matrix interaction, and nutritional and hormonal signals, as discussed above (*see* Section 3.4.1). Some signaling is contact-mediated via cell adhesion molecules (*see* Section 3.2), but signaling can also result from soluble, diffusible factors. Signals that reach the cell from another tissue via the systemic vasculature are called *endocrine*, and those that diffuse from adjacent cells without entering the bloodstream are called *paracrine*. It is useful to recognize that some soluble signals arise in, and interact with, the same type of cell. I will call this *homotypic paracrine*, or *homocrine*, signaling (Fig. 3.7). Signals that arise in a cell type different from the responding cells are

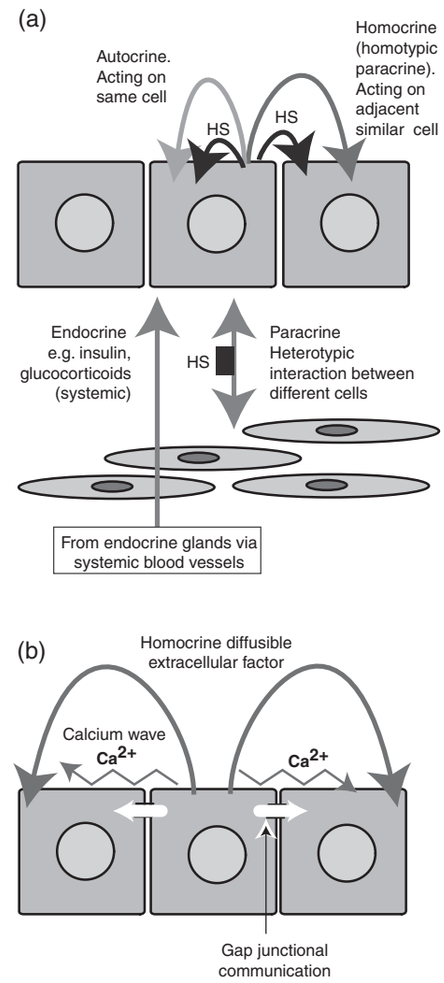

Fig. 3.7. Cell Interaction and Signaling. Routes of interaction among cells. (a) Factors influencing the behavior of a cell include endocrine hormones from the vasculature, paracrine factors from the stroma, homocrine factors from adjacent similar cells, and autocrine factors from the cell itself. Matrix, soluble, and cell-associated heparan sulfate (HS) may help the activation, stabilization, and/or translocation of paracrine factors. (b) Uniformity of response in target tissue is improved by gap junctional communication, by calcium signaling, and, possibly, by homocrine factors from the stimulated cell.

heterotypic paracrine and will be referred to simply as *paracrine* in any subsequent discussion. A cell can also generate its own signaling factors that bind to its own receptors, and this is called *autocrine* signaling.

Although all of these forms of signaling occur *in vivo*, under normal conditions with basal media *in vitro*, only autocrine and homocrine signaling will occur. The failure of many cultures to plate with a high efficiency at low cell densities may be due, in part, to the dilution of one or more autocrine and homocrine factors, and this is part of the rationale in using conditioned medium (*see* Protocol 14.2) or feeder layers (*see* Protocol 14.3) to enhance plating efficiency. As the maintenance and proliferation of specialized cells, and the induction of their differentiation, may depend on paracrine and endocrine factors, these must be identified and added to differentiation medium (*see* Sections 17.7.1, 17.7.2). However, their action may be quite complex as not only may two or more factors be required to act in synergy [*see*, e.g., McCormick and Freshney, 2000], but, in trying to simulate cell–cell interaction by supplying exogenous paracrine factors, it is necessary to take into account that the phenotype of interacting cells, and hence the factors that they produce and the time frame in which they are produced, will change as a result of the interaction. Heterotypic combinations of cells may be, initially at least, a simpler way of providing the correct factors in the correct matrix microenvironment, and analysis of this interaction may then be possible with blocking antibodies or antisense RNA.

3.6 ENERGY METABOLISM

Most culture media contain 4–20 mM glucose, which is used mainly as a carbon source for glycolysis, generating lactic acid as an end product. Under normal culture conditions (atmospheric oxygen and a submerged culture), oxygen is in relatively short supply. In the absence of an appropriate carrier, such as hemoglobin, raising the O_2 tension will generate free radical species that are toxic to the cell, so O_2 is usually maintained at atmospheric levels. This results in anaerobic conditions and the use of glycolysis for energy metabolism. However, the citric acid cycle remains active, and it has become apparent that amino acids—particularly glutamine—can be utilized as a carbon source by oxidation to glutamate by glutaminase and entry into the citric acid cycle by transamination to 2-oxoglutarate [Reitzer et al., 1979; Butler & Christie, 1994]. Deamination of the glutamine tends to produce ammonia, which is toxic and can limit cell growth, but the use of dipeptides, such as glutamyl-alanine or glutamyl-glycine, appears to minimize the production of ammonia and has the additional advantage of being more stable in the medium (e.g., Glutamax, Invitrogen).

3.7 INITIATION OF THE CULTURE

Primary culture techniques are described in detail later (*see* Chapter 12). Briefly, a culture is derived either by the outgrowth of migrating cells from a fragment of tissue or by enzymatic or mechanical dispersal of the tissue. Regardless of the method employed, primary culture is the first in a series of selective processes (Table 3.2) that may ultimately give rise to a relatively uniform cell line. In primary explantation (*see* Section 12.3.1), selection occurs by virtue of the cells' capacity to migrate from the explant, whereas with dispersed cells, only those cells that both survive the disaggregation technique and adhere to the substrate or survive in suspension will form the basis of a primary culture. If the primary culture is maintained for more than a few hours, a further selection step will occur. Cells that are capable of proliferation will increase, some cell types will survive but not increase, and yet others will be unable to survive under the particular conditions of the culture. Hence, the relative proportion of each cell type will change and will continue to do so until, in the case of monolayer cultures, all the available culture substrate is occupied. It should be realized that primary cultures, although suitable for some studies such as cytogenetic analysis, may be unsuitable for other studies because of their instability. Both cell population changes and adaptive modifications within the cells are occurring continuously throughout the culture, making it difficult

TABLE 3.2. Selection in Cell Line Development

	Factors influencing selection	
Stage	Primary explant	Enzymatic disaggregation
Isolation	Mechanical damage	Enzymatic damage
Primary culture	Adhesion of explant; outgrowth (migration), cell proliferation	Cell adhesion and spreading, cell proliferation
First subculture	Trypsin sensitivity; nutrient, hormone, and substrate limitations; proliferative ability	
Propagation as a cell line	Relative growth rates of different cells; selective overgrowth of one lineage Nutrient, hormone, and substrate limitations Effect of cell density on predominance of normal or transformed phenotype	
Senescence; transformation	Normal cells die out; transformed cells overgrow	

to select a period when the culture may be regarded as homogeneous or stable.

After *confluence* is reached (i.e., all the available growth area is utilized and the cells make close contact with one another), cells whose growth is sensitive to contact inhibition and density limitation of cell proliferation (*see* Section 18.5.2) will stop dividing, while any transformed cells, which are insensitive to density limitation, will tend to overgrow. Keeping the cell density low (e.g., by frequent subculture) helps to preserve the normal phenotype in cultures such as mouse fibroblasts, in which spontaneous transformants tend to overgrow at high cell densities [Todaro & Green, 1963].

Some aspects of specialized function are expressed more strongly in primary culture, particularly when the culture becomes confluent. At this stage, the culture will show its closest morphological resemblance to the parent tissue and retain some diversity in cell type.

3.8 EVOLUTION OF CELL LINES

After the first subculture, or passage (Fig. 3.8), the primary culture becomes known as a *cell line* and may be propagated and subcultured several times. With each successive subculture, the component of the population with the ability to proliferate most rapidly will gradually predominate, and nonproliferating or slowly proliferating cells will be diluted out. This is most strikingly apparent

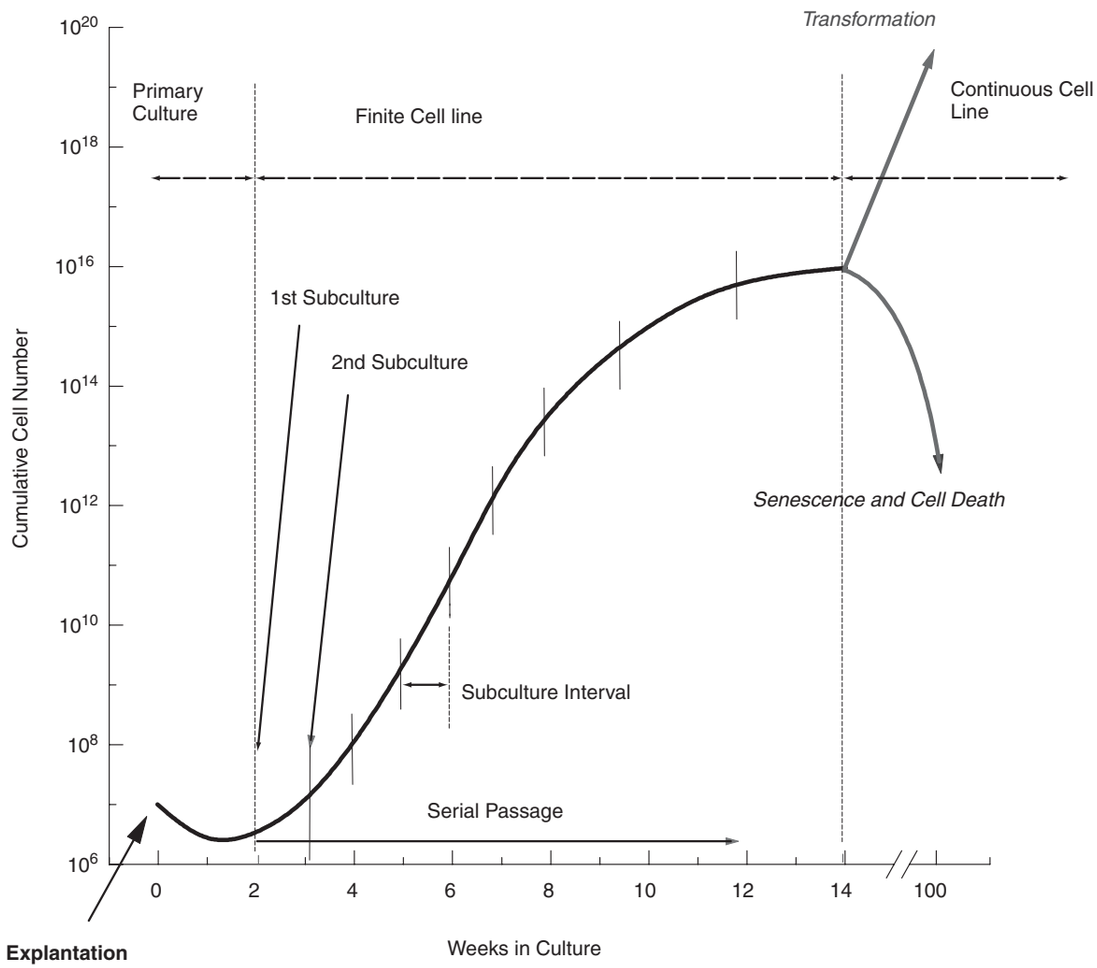

Fig. 3.8. Evolution of a Cell Line. The vertical (*Y*) axis represents total cell growth (assuming no reduction at passage) for a hypothetical cell culture. Total cell number (cell yield) is represented on this axis on a log scale, and the time in culture is shown on the *X*-axis on a linear scale. Although a continuous cell line is depicted as arising at 14 weeks, with different cells it could arise at any time. Likewise, senescence may occur at any time, but for human diploid fibroblasts it is most likely to occur between 30 and 60 cell doublings, or 10 to 20 weeks, depending on the doubling time. Terms and definitions used are as in the glossary. (After Hayflick and Moorhead [1961].)

after the first subculture, in which differences in proliferative capacity are compounded with varying abilities to withstand the trauma of trypsinization and transfer (*see* Section 13.1).

Although some selection and phenotypic drift will continue, by the third passage the culture becomes more stable and is typified by a rather hardy, rapidly proliferating cell. In the presence of serum and without specific selection conditions, mesenchymal cells derived from connective tissue fibroblasts or vascular elements frequently overgrow the culture. Although this has given rise to some very useful cell lines (e.g., WI-38 human embryonic lung fibroblasts [Hayflick and Moorhead, 1961], BHK21 baby hamster kidney fibroblasts [Macpherson and Stoker, 1962], COS cells [Gluzman, 1981], CHO cells [Puck et al., 1958] (*see* Table 13.1), and perhaps the most famous of all, the L-cell, a mouse subcutaneous fibroblast treated with methylcholanthrene [Earle et al., 1943; Sanford et al., 1948]), this overgrowth represents one of the major challenges of tissue culture since its inception—namely, how to prevent the overgrowth of the more fragile or slower-growing specialized cells such as hepatic parenchyma or epidermal keratinocytes. Inadequacy of the culture conditions is largely to blame for this problem, and considerable progress has now been made in the use of selective media and substrates for the maintenance of many specialized cell lines (*see* Section 10.2.1, Chapter 23).

3.8.1 Senescence

Normal cells can divide a limited number of times; hence, cell lines derived from normal tissue will die out after a fixed number of population doublings. This is a genetically determined event involving several different genes and is known as *senescence*. It is thought to be determined, in part, by the inability of terminal sequences of the DNA in the telomeres to replicate at each cell division. The result is a progressive shortening of the telomeres until, finally, the cell is unable to divide further [Bodnar et al., 1998]. Exceptions to this rule are germ cells, stem cells, and transformed cells, which often express the enzyme telomerase, which is capable of replicating the terminal sequences of DNA in the telomere and extending the life span of the cells, infinitely in the case of germ cells and some tumor cells (*see also* Section 18.4.1).

3.9 THE DEVELOPMENT OF CONTINUOUS CELL LINES

Some cell lines may give rise to continuous cell lines (*see* Fig. 3.7). The ability of a cell line to grow continuously probably reflects its capacity for genetic variation, allowing subsequent selection. Genetic variation often involves the deletion or mutation of the p53 gene, which would normally arrest cell cycle progression, if DNA were to become mutated, and overexpression of the telomerase gene. Human fibroblasts remain predominantly euploid throughout their life span in culture and never give rise to continuous cell lines [Hayflick and Moorhead, 1961], whereas mouse fibroblasts and cell cultures from a variety of human and animal tumors often become aneuploid in culture and frequently give rise to continuous cultures. Possibly the condition that predisposes most to the development of a continuous cell line is inherent genetic variation, so it is not surprising to find genetic instability perpetuated in continuous cell lines. A common feature of many human continuous cell lines is the development of a subtetraploid chromosome number (Fig. 3.9). The alteration in a culture that gives rise to a continuous cell line is commonly called *in vitro transformation* (*see* Chapter 18) and may occur spontaneously or be chemically or virally induced (*see* Sections 18.2, 18.4). The word *transformation* is used rather loosely and can mean different things to different people. In this volume, *immortalization* means the acquisition of an infinite life span and *transformation* implies an additional alteration in growth characteristics (anchorage independence, loss of contact inhibition and density limitation of growth) that will often, but not necessarily, correlate with tumorigenicity.

Continuous cell lines are usually *aneuploid* and often have a chromosome number between the diploid and tetraploid values (*see* Fig. 3.9). There is also considerable variation in chromosome number and constitution among cells in the population (*heteroploidy*) (*see also* Section 18.3.) It is not clear whether the cells that give rise to continuous lines are present at explantation in very small numbers or arise later as a result of the transformation of one or more cells. The second alternative would seem to be more probable on cell kinetic grounds, as continuous cell lines can appear quite late in a

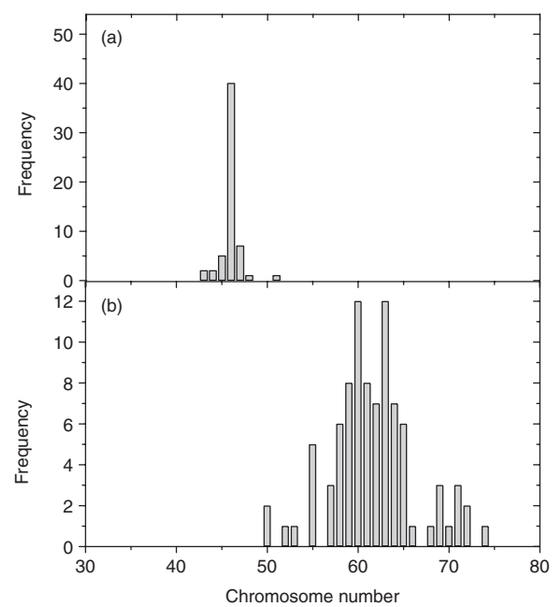

Fig. 3.9. Chromosome Numbers of Finite and Continuous Cell Lines. (a) A normal human glial cell line. (b) A continuous cell line from human metastatic melanoma.

culture's life history, long after the time it would have taken for even one preexisting cell to overgrow. The possibility remains, however, that there is a subpopulation in such cultures with a predisposition to transform that is not shared by the rest of the cells.

The term *transformation* has been applied to the process of formation of a continuous cell line partly because the culture undergoes morphological and kinetic alterations, but also because the formation of a continuous cell line is often accompanied by an increase in tumorigenicity. A number of the properties of continuous cell lines, such as a reduced serum requirement, reduced density limitation of growth, growth in semisolid media, aneuploidy (*see also* Table 18.1 and Plate 14), and more, are associated with *malignant* transformations (*see* Section 18.6). Similar morphological and behavioral changes can also be observed in cells that have undergone virally or chemically induced transformation.

Many (if not most) normal cells do not give rise to continuous cell lines. In the classic example, normal human fibroblasts remain euploid throughout their life span and at crisis (usually around 50 generations) will stop dividing, although they may remain viable for up to 18 months thereafter. Human glia [Pontén & Westermark, 1980] and chick fibroblasts [Hay & Strehler, 1967] behave similarly. Epidermal cells, on the other hand, have shown gradually increasing life spans with improvements in culture techniques [Rheinwald & Green, 1977; Green et al., 1979] and may yet be shown capable of giving rise to continuous growth. Such growth may be related to the self-renewal capacity of the tissue *in vivo* and successful propagation of the stem cells *in vitro* (*see* Section 3.10). Continuous culture of lymphoblastoid cells is also possible [Gjerset et al., 1990] by transformation with Epstein–Barr virus.

3.10 ORIGIN OF CULTURED CELLS

Because most people working under standard conditions do so with finite or continuous proliferating cell lines, it is important to consider the cellular composition of the culture. The capacity to express differentiated markers under the influence of inducing conditions may mean either that the cells being cultured are mature and only require induction to continue synthesizing specialized proteins or that the culture is composed of precursor or stem cells that are capable of proliferation but remain undifferentiated until the correct inducing conditions are applied, whereupon some or all of the cells mature and become differentiated. It may be useful to think of a cell culture as being in equilibrium between multipotent stem cells, undifferentiated but committed precursor cells, and mature differentiated cells (*see* Fig. 3.6) and to suppose that the equilibrium may shift according to the environmental conditions. Routine serial passage at relatively low cell densities would promote cell proliferation and constrain differentiation, whereas high cell densities, low serum, and the appropriate hormones would promote differentiation and inhibit cell proliferation.

The source of the culture will also determine which cellular components may be present. Hence, cell lines derived from the embryo may contain more stem cells and precursor cells and be capable of greater self-renewal than cultures from adults. In addition, cultures from tissues undergoing continuous renewal *in vivo* (epidermis, intestinal epithelium, hematopoietic cells) may still contain stem cells, which, under the appropriate conditions, will have a prolonged life span, whereas cultures from tissues that renew solely under stress (fibroblasts, muscle, glia) may contain only committed precursor cells with a limited life span.

Thus the identity of the cultured cell is defined not only by its lineage *in vivo* (hematopoietic, hepatocyte, glial, etc.), but also by its position in that lineage (stem cell, precursor cell, or mature differentiated cell). Although progression down a differentiation pathway has been thought of as irreversible, the concept of commitment is now being questioned [Kondo & Raff, 2004; Le Douarin et al., 2004] and some precursor cells may be able to convert or revert to stem cell status and redifferentiate along the same or a different lineage.

When cells are cultured from a neoplasm, they need not adhere to these rules. Thus a hepatoma from rat may proliferate *in vitro* and still express some differentiated features, but the closer they are to those of the normal phenotype, the more induction of differentiation may inhibit proliferation. Although the relationship between position in the lineage and cell proliferation may become relaxed (though not lost—B16 melanoma cells still produce more pigment at a high cell density and at a low rate of cell proliferation than at a low cell density and a high rate of cell proliferation), transfer between lineages has not been clearly established (*see* Section 17.4).