Introduction

There are many different types of cells that constitute the tissues and form the various organs in a mammal. They all arise from the same fertilized egg and then differentiate into cells with vastly different properties, using the same set of genetic information but arriving at very different destinies. Over a century ago, scientists began to isolate cells from explanted tissues. In Chapter 1 we briefly discussed the enabling advances that made cell culture a research tool and production host, including the development of media and its membrane sterilization and cell trypsinization and passaging. Most cells that outgrew from tissues could only be cultured for a very small number of generations, but eventually some cells were successfully established that could be passaged over many generations. After cryopreservation methods were established, cells could be "banked" for future use, allowing for the generation of the reproducible results that are essential to research and manufacturing.

Most cells established in the early decades of cell culture were derived from tumors. Those cells appeared morphologically different from primary cells that had grown out of normal tissues. Even those that were isolated originally from normal tissues and could be continuously passaged appeared more like cells that had originated from tumors. With increasing knowledge of growth factors and extracellular matrices, normal and differentiated cells began to be cultured in the 1970s. The isolation of adult stem cells and then mouse embryonic pluripotent stem cells, concurrently with the wide availability of many more growth factors and cytokines, began to change the landscape of cell culture for research. The

drive to produce therapeutic proteins in large quantities in the 1990s changed our view of cells. They are now the primary workhorse for biomanufacturing. This chapter will discuss the key aspects of a cell's structural components that affect growth and the cell's capability to produce the protein product.

Tissue Cells and Their Isolation

Cell Lines, Cell Strains, and Senescence

The vast majority of cells isolated from tissues of mammals are anchorage-dependent, meaning that they require surface adhesion in order to multiply. They are typically isolated from tissues by an enzymatic dissociation of the tissue (Figure 2.1). After dissociation and the removal of undissociated chunks, cells are plated on a compatible surface overlaid with media. The cell clumps in media suspension attach to the surface and gradually some cells grow out from the tissue clumps. Subsequently, cells extend their body length, spread, and begin to multiply. As they begin to cover the entire surface area the growth rate slows down, eventually forming a "monolayer" of cells over the surface. Upon reaching confluence, cell division stops. While the cell bodies of neighboring cells may cross each other, their nuclei never overlap. This is called contact inhibition of cell growth. The contact inhibited cell can be dissociated from the surface by treatment with trypsin. After being plated on a larger surface and provided with fresh medium, cell growth resumes until confluence is again reached. This process can be repeated to expand the population. Each round of detachment and expansion is called a "passage." The number of cell doublings taking place in each passage is determined by the split ratio, or the ratio of surface area expanded in each passage (see reference 1 for further reading).

Cells isolated directly from tissues are called primary cells (Panel 2.1). Most primary cells stop growing after a very small number of cell doublings. This is especially true for cells isolated from specialized tissues, such as from the liver and ovaries. They often quickly lose their typical tissue-associated characteristics in culture.

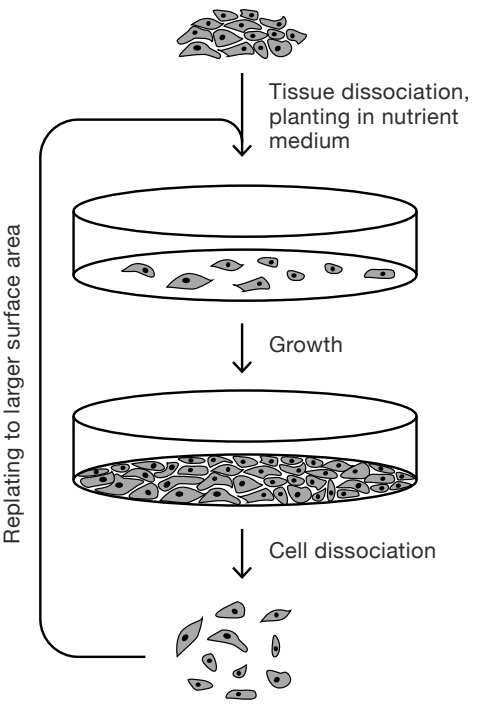

Figure 2.1. Anchorage dependency and contact inhibition in cultured normal diploid cells.

Panel 2.1. Cell Types by Their Proliferative Potential *in Vitro*

Primary Cells
- Isolated directly from tissue with very limited capacity of cell expansion *in vitro*

Cell Strain
- Cells outgrown from isolated tissues that can be passaged and still maintain their normal behavior and diploid chromosome makeup
- Eventually they reach senescence

Continuous Cell Line
- Cells isolated from cancer or normal tissue that can be serially expanded without reaching senescence
- Not diploid and often lose their normal morphology

Multipotent Stem Cells
- Stem cells that can differentiate into different types of the same lineage

Pluripotent Stem Cells
- Stem cells that can differentiate into all different cell types as an adult
- Either embryonic stem cells or induced pluripotent stem cells (iPSCs)

Panel 2.2. Quiescent Cells and Growth Potential

- In mammals, most cells are quiescent or at a growth-arrested state. Only some cell types in some tissues, such as epithelial tissue in the intestines, divide actively.
- Cells that can be isolated and cultured *in vitro* have undergone changes to overcome growth arrest. They have somewhat different physiological states than their quiescent counterparts even if they appear normal.
- Cancer cells proliferate *in vivo* and are more amenable to culture growth

Most cells in tissues *in vivo* are quiescent, meaning that they are not dividing (Panel 2.2). The quiescent state is not merely the passive result of a lack of conditions necessary for growth, such as a missing essential nutrient. Rather, the quiescent state is imposed on the cell by the regulation of the organism. For example, stem cells in our body are in a quiescent state most of the time, as a result of cell cycle regulation through cyclin-dependent kinase inhibitors, transcription factors, and tumor suppressors. Cells that grow out from tissue explant have undergone changes in their cell growth control mechanism that enable them to proliferate in culture. In some cases, the cells outgrown from isolated tissue can be expanded serially over many passages and still retain normal morphology and cell behavior. They can even be cryopreserved in liquid nitrogen and be "banked" for future research, distribution, or manufacturing purposes. However, such repeated passaging and cell expansion in culture cannot be continued indefinitely. Eventually, senescence is reached. Before senescence, these cells are morphologically normal. Their karyotype is diploid, i.e., having two sets of normal chromosomes. Virtually all normal diploid cells derived from mammal tissues have a limited life span in culture, with the exception of embryonic stem cells and induced pluripotent stem cells.

In contrast to tissue cells, cancer cells proliferate *in vivo*. They typically carry a variety of mutations that allow them to bypass the growth control mechanism that would normally keep them in a quiescent state. Therefore, they are more readily isolated from tumor tissue for culture *in vitro*. Sometimes, lines of cells can also be established from normal tissues by "immortalization" through viral or oncogene

transformation. Those cells that can be grown continuously without senescence typically do not have the same morphology as their normal counterparts. Their karyotype is not diploid and their growth is not contact inhibited. With adequate supplies of nutrients and growth factors, they can even overlay each other to form multiple layers of cells. Such cells include commonly used industrial cell lines like CHO cells from Chinese hamster ovary, Vero cells from green monkey kidney, and human kidney cell HEK293.

Without using special culture conditions to enrich differentiated cells, fibroblast cells are the predominant cell type isolated from normal mammalian tissues. Fibroblasts isolated from a mouse embryo can be cultured *in vitro* for about 60 doublings (Figure 2.2). As that limit approaches, the cells begin to fail to reach confluence and eventually cease to grow. This senescence, or a limit in the proliferating potential of these cells, is called "Hayflick's phenomenon." It is a common phenomenon for all normal diploid cells (again, with embryonic stem cells and induced pluripotent stem cells as exceptions) obtained from vertebrates.

In a historic experiment carried out half a century ago, the continued passaging of mouse fibroblast cells beyond crisis gave rise to a small fraction of survivors. These cells eventually grew, expanded, and could be cultured continuously *in vitro* without a limited life span. They were given the name 3T3 because the cells were passaged every three days by expanding at 3 x 10^5 cells per 20 cm^2 dish. These cells appear normal and are subjected to contact inhibition of growth under typical culture conditions.

However, unlike the mouse fibroblasts of the pre-crisis period, 3T3 cells have an abnormal, non-diploid set of chromosomes. Cells that succumb to Hayflick's constraint (e.g., those that are diploid and have a

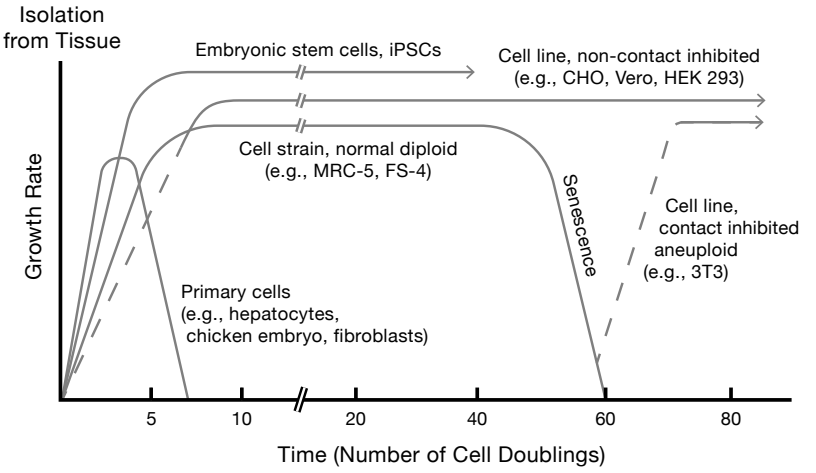

Figure 2.2. Life spans and growth rates of different cell types.

limited life span) are called "cell strains." The cells that can grow in culture indefinitely are referred to as "cell lines," including those reestablished after passing the crisis and derived directly from normal or tumor tissues. In general, cell lines are aneuploid (do not have a normal set of chromosomes).

Cells that are subject to senescence thus appear to "count" their number of doublings. Senescence is regulated by cellular events and is often thought to be related to aging. It is essentially a growth arrest, but one that differs from the quiescent state. Cells in a quiescent state can revert to their growth phase, but cells entering senescence undergo an irreversible growth arrest. Senescence may also occur in response to stress or overexpression of oncogene, or be induced by telomere shortening. Telomeres are special repetitive sequences at the end of chromosomes. They are not replicated by DNA polymerase during DNA replication but are synthesized by telomerase. The DNA polymerase reaction does not accurately reproduce the number of tandem repeats of the telomeres. There is thus much variation in telomere length among cells. As the number of passages increases, telomeres become shorter unless they are repaired by telomerase. For instance, in embryonic stem cells, telomerase activity is high in order to maintain the telomere length. Unlike cell strains, embryonic stem cells do not exhibit senescence.

Stem Cells and Differentiated Cells

Cells isolated from normal or cancerous differentiated tissues often retain their differentiated properties to different extents depending on the chemical and physical environment. Many differentiated cells isolated from cancerous tissues, including HepG2 cells (from hepatocellular carcinoma), Jurkat cells (human T lymphocyte from leukemia), and PC12 (neuronal cells isolated from pheochromocytoma of the rat adrenal medulla, can be induced to form dendrites in culture) have been valuable in biomedical research for decades. They are all continuous cell lines. Although they retain some differentiated phenotypes of the tissue that they were derived from, many important tissue characteristics are lost. The advances in the research tools for cell characterization and growth factors in the past three decades have allowed for the cultivation of various differentiated cells from normal tissues, including endothelial cells, keratinocytes, melanocytes, mononuclear cells, and smooth muscle cells. These primary tissue cells retain many tissue specific activities and are valuable not only in research but also potentially for tissue repair or regeneration. However, as will be discussed later in this chapter, most differentiated cells *in vivo* are at a G_0 phase of cell culture and are not proliferating. Cells isolated from those tissues have very limited proliferative potential and show phenotypic instability in culture. For possible regenerative

medicine applications, differentiated cells isolated from tissues are not sustainable cell sources.

Stem cells must be capable of self-renewal (i.e., the ability to make more of themselves) and differentiation into their target cells. They exist in many adult tissues and are quiescent in their niche, expanding and differentiating only when needed. Some adult stem cells have been isolated and grown in culture for decades, including hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) isolated from bone marrow. They have limited proliferative potential, and after some generations in culture they lose their renewal and differentiation capacity. These adult stem cells are multipotent, meaning that they can differentiate into multiple, but a limited number of, cell lineages. Even with a less-than-unlimited proliferative potential, adult stem cells have the capacity to give rise to a large number of differentiated cells (such as bone and muscle cells from MSCs) for allogeneic applications.

In contrast to adult stem cells, embryonic stem cells isolated in a very early stage of embryo development have a virtually unlimited self-renewal capacity in culture and are pluripotent, meaning that they can differentiate into cell types of all adult tissues. They do not undergo senescence and have tremendous potential in regenerative applications. However, as they are derived from fertilized human eggs, their use is ethically controversial. To alleviate these ethical concerns, a process was devised to reprogram adult somatic cells into an undifferentiated embryonic state. These induced pluripotent stem cells (iPSCs) can be obtained by epigenetic reprogramming of adult tissue cells like fibroblasts through the introduction of four exogenous genes (OCT4, SOX2, KLF4, and c-Myc (OSKM)). The iPSCs have a similar potential to differentiate into cells of different lineages as ESCs. Both ESCs and iPSCs can be directed to differentiate into various types of cells, including hematopoietic stem cells, hepatocytes (liver cells), and pancreatic cells by using series of cocktails of growth factors to mimic *in vivo* differentiation conditions.

Cells for Biologics Manufacturing

The cells commonly used for the production of biologics are derived from different tissues of different species. Cells isolated from different species differ in their chromosome number and genome sequence. However, at a physiological and transcriptome level, cells from the same tissue of different species are strikingly similar. Their similarity is much greater than with different cell types from the same species. For example, chicken embryo fibroblasts look morphologically very similar to human fibroblasts from the lung or foreskin, while the epithelial MDCK cells look rather different from dog fibroblasts even though they are both derived from the same species.

Table 2.1. Cells Commonly Used in Bioprocessing

Cell name	*Species*	*Cell type*	*Tissue isolated*
WI-38	Human	Fibroblast	Lung
MRC-5	Human	Fibroblast	Lung
FS-4	Human	Fibroblast	Foreskin
HEK293	Human	Epithelial	Kidney
Vero	Monkey	Epithelial	Kidney
MDCK	Dog	Epithelial	Kidney
NS/SP2/0	Mouse	Lymphoid	Myeloma
CHO	Chinese hamster	Epithelial	Ovary
BHK	Syrian hamster	Fibroblast	Kidney

Panel 2.3. Cell Types of Different Tissues

- Among the ~200 different types of cells, fibroblasts, epithelial cells, and myeloma cells are the most frequently used cell types in biologics production
- Cells in culture bear closer physiological and morphological characteristics to the tissue they were derived from than to their species

Even though there are about two hundred types of cells in a vertebrate animal, most cells that are used for the production of biologics are either epithelial or fibroblastic in nature (Table 2.1, Panel 2.3). These two cell types are generally amenable to isolation from tissues and to *in vitro* culture, as demonstrated during the early explorations on tissue cell isolation more than half a century ago. NS0 and CHO are the two prominent host cell lines used for therapeutic recombinant protein production. They exhibit different behaviors and were derived from two different tissues and two different species. CHO cells were isolated from the ovary of a Chinese hamster, NS0 cells from a mouse myeloma. Cells used for recombinant protein production are primarily epithelial and lymphatic. Both fibroblasts and epithelial cells are also frequently used for viral vaccine production. Cell line selection for recombinant protein production versus vaccine production are based on fundamentally different needs. The former demands easy genetic manipulation and amenability to high protein secretion, while the latter requires cells with the matching tropism of the virus and a minimal antiviral response.

Epithelial cells line the "boundary" of tissues, while fibroblasts make up a larger part of the connective tissue. Epithelial cells form tightly connected sheets, which often become damaged, die, and are replenished by "new" ones. Fibroblasts are mostly quiescent. They migrate into wounds and begin to grow only when they are stimulated by various cues. The terminally differentiated plasma cells from which myeloma cells are derived secrete antibodies against a particular antigen. The antibodies are needed only for a limited period of time after the host's exposure to the antigen. These cells undergo apoptosis two to three weeks after their differentiation into active antibody-secreting cells so that the host does not continue to have a large quantity of unneeded antibody molecules in circulation. Many native characteristics of the original-tissue cell are often still evident in tissue-derived cell lines in culture.

Cell Membrane

An antiquated but previously common-held belief regarding the growing of mammalian cells in a bioreactor was that cells were extreme fragile and vulnerable to mechanical stress because the only thing preventing cellular content from dissolving into the aqueous environment was the lipid bilayer membrane. Yet, in a modern manufacturing plant, cells thrive in bioreactors of tens of cubic meters in volume under highly turbulent conditions. The cytoplasmic membrane surrounding a cell is much more than a mere double layer of lipids and the integrity of a cell is not only dictated by its membrane wrapping.

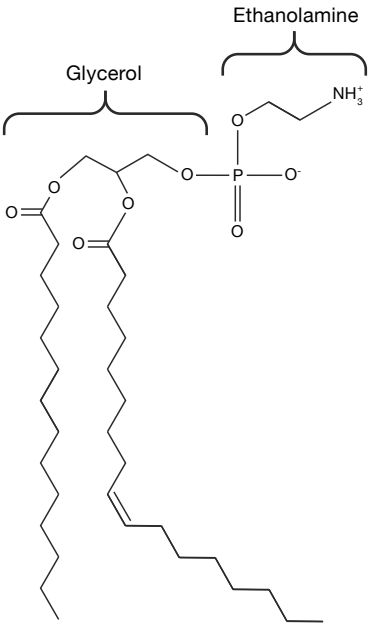

Figure 2.3. A phospholipid molecule with glycerol conjugated with an ethanolamine, a saturated fatty acid, and an unsaturated fatty acid.

Lipid Bilayer

The lipids that make up the lipid bilayer are amphipathic phospholipids. Each phospholipid molecule consists of a glycerol backbone linking a hydrophilic head group that includes a charged phosphate group and a hydrophobic tail group consisting of two fatty acids (Figure 2.3). The fatty acids at the hydrophobic tail provide the hydrophobic interactions necessary to form the ordered structure of a membrane at a mild temperature. When suspended in an aqueous solution, amphipathic molecules can form micelles. In such micelles, the hydrophilic heads face outward and the hydrophobic tails project inward. The organization of a micelle makes it easy to enclose hydrophobic molecules inside while not having an aqueous environment both inside and outside. In contrast, a sphere formed by a lipid bilayer membrane has the hydrophobic tails projecting toward the middle of the bilayer and the hydrophilic heads on the external as well as the internal surfaces. It can readily have an aqueous environment both inside and outside (Figure 2.4).

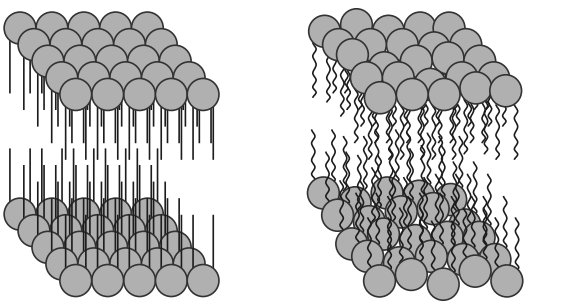

Figure 2.4. Lipid bilayer membrane at a gel state and a liquid crystal state.

A lipid bilayer membrane behaves like a fluid (Panel 2.4). If the lipid molecules in a specific location were to be labeled with a fluorescent

Panel 2.4. Properties of a Lipid Bilayer

- Fluid
- As temperature decreases, the bilayer transitions from a fluid state to a gel state
- The extent of unsaturation and length of fatty acids affects the transition temperature of the membrane
- The magnitude of diffusion of various solutes in the cell membrane resembles that of a liquid

Panel 2.5. Lipid Bilayer Composition

Phospholipids
- Constitute the majority (35–70%)

Glycolipids
- Neutral glycolipids (e.g., galactocerebroside)

Gangliosides
- Have sialic acids

Types of Phospholipids
- Glycerol as backbone
  - Phosphatidyl ethanol amine
  - Phosphatidyl serine
  - Phosphatidyl choline
- Serine as backbone
  - Ceramide
  - Sphingomyelin

dye, the fluorescence would disperse quickly due to molecular diffusion. The lateral diffusion coefficient of a phospholipid molecule in a bilayer membrane is about 10^{-8} cm^2/s. Gas species diffuse about equally fast in a lipid bilayer as in water. Even large protein molecules diffuse in a lipid bilayer membrane.

Although a lipid bilayer membrane is a fluid, by forming a tightly packed, ordered structure it becomes a very good barrier that keeps most molecules from freely passing into or out of the cell. A lipid molecule does not flip-flop (i.e., change its side of the lipid bilayer) without the aid of a membrane-bound phospholipid translocator. The permeability of most biological molecules across a lipid bilayer membrane is rather low. Even the smallest nutrients, like glucose and simple amino acids, cannot pass through frequently enough to support cell growth.

All major biological macromolecules (e.g., DNA, proteins, polysaccharides) are biopolymers made of covalently-bonded monomers. A lipid bilayer membrane is not a polymer, but rather an assembly of lipid monomers. The noncovalent nature of the cell membrane allows it to expand, shrink, break, change shape, and fuse rapidly. As cells grow, the cell membrane can expand rapidly, dynamically changing its composition and shape to meet cellular needs without breaking down many covalent bonds as in polymeric biomolecules.

The lipid bilayer membrane makes up not only the cytoplasmic membrane enwrapping the cell. It also envelops various organelles to compartmentalize regions in the cell for specialized functions. Major organelles in a cell include the nucleus, mitochondrion, endoplasmic reticulum (ER), Golgi apparatus, and lysosome. Many of these organelles are in a constant dynamic process of membrane budding and fusion. For example, in trafficking between organelles and in protein secretion, the "cargo" is carried inside membrane vesicles while transiting from one organelle to another.

Three types of lipids make up the lipid bilayer membrane in cells and organelles: phospholipids, glycolipids, and gangliosides (phospholipids

being the most common) (Panel 2.5). There are several different types of phospholipids. The most abundant type is glycerophospholipid, which uses glycerol as the backbone. Its 3-hydroxyal group of glycerol is linked to a phosphate group, to which an ethanolamine or serine is attached. The other two hydroxyl groups of glycerol are linked to two fatty acids through an ester bond. Typically, one of those two fatty acids is saturated and the other is unsaturated, with a cis double bond in between C14 and C24 (Panel 2.6). The degree of unsaturation affects the packing of the lipid bilayer. Saturated fatty acids allow more dense packing, while the double bonds in the unsaturated fatty acids create kinks, which reduce packing and increase the membrane fluidity.

Like other lipids, the lipid bilayer membrane undergoes a phase transition from a liquid (or liquid crystal) phase to a gel phase at a relatively moderate temperature (Figure 2.4). A lipid bilayer can be in a gel state or in a liquid crystalline state depending on the temperature and degree of hydration. A lipid bilayer's phase transition temperature is affected by its composition of fatty acid and cholesterol. As temperature decreases, the lipid bilayer changes from a liquid-crystalline state to crystalline (or gel) state. A higher content of shorter, unsaturated fatty

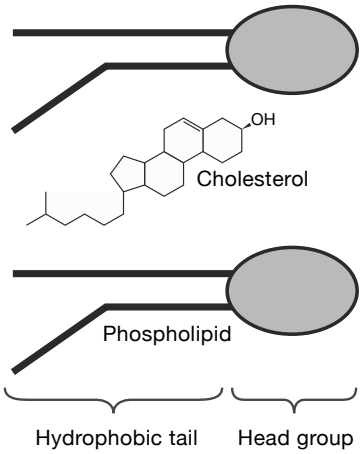

Figure 2.5. Schematic diagram of a cholesterol molecule interacting with two phospholipid molecules in one leaflet of a lipid bilayer membrane.

Panel 2.6. Lipid Composition and Membrane Fluidity

- One saturated, one cis-unsaturated (C14-C24) typically constitute the tail of the phospholipid
- Fatty acids (the tail group) on the lipid affect the packing of lipids in the bilayer membrane. Saturated fatty acids allow more dense packing; double bonds in unsaturated fatty acids create kinks, reduce packing, and increase fluidity.
- Cholesterol has a small head polar group linked to a rigid planar region of steroid rings followed by a more flexible non-polar tail. It interacts with phospholipids to stabilize the region closer to the head group as well as to make the lipid bilayer less inclined to become crystalline. Overall, it increases the membrane permeability to small compounds and makes the membrane less fluid.

Table 2.2. Biochemical Composition of a Hepatocyte Plasma Membrane

Total lipids	*Total proteins*	*Protein / lipid mass ratio*	*Cholesterol / phospholipid molar ratio*	*Cholesterol in total lipids*	*Phospholipids in total lipids*
30–40% (by mass)	50–60% (by mass)	1:2	0.4–0.8	12–20%	50–70%

Adapted from *The Liver: Biology & Pathology*, 4th Ed., p. 78 (2001).

acids increases the fluidity of the lipid bilayer and decreases its phase transition temperature.

Another molecule that plays a key role in the membrane properties of animal cells is cholesterol (Figure 2.5). Cholesterol has a small polar hydroxyl head group linked to a rigid planar region of steroid rings that are further linked to a more flexible non-polar tail. Cholesterol interacts with phospholipids to stabilize the region closer to the head group and to make the lipid bilayer less inclined to become crystalline. Overall, cholesterol increases the membrane permeability to small compounds and makes the membrane less fluid. Cholesterol content varies in different lipid bilayer membranes. Its level in the cytoplasmic membrane is high, but it is very low or absent in the organelle membranes.

Membrane Proteins

A typical biological membrane has ~50% lipids and ~50% proteins, by mass (Table 2.2, Panel 2.7). In terms of the number of molecules, the lipid-protein ratio is about 50:1, since proteins have much higher molecular weight than lipids. The protein content of a membrane is greatly affected by the tissue and organelle of origin. The mitochondrial membrane, through which many molecules (e.g., amino acids, pyruvate, various ions, and many other proteins) pass at a high flux, has a high protein content of about 75% by mass. On the other hand, the myelin membrane, which serves as a protective sheath between the nerve cell and its surroundings, has a low protein content of about 25%.

Panel 2.7. Membrane Proteins

- A typical biological membrane has ~50% proteins by mass; in terms of molecules, lipid : protein = 50 : 1
- A metabolically active mitochondrion's membrane is 75% protein
- Na^+/K^+ ATPase acts as a pump, using ATP to pump 3 Na^+ out of and 2 K^+ into the cell
- The electric potential across the plasma membrane is about −80mV.

Lipid bilayer membranes separate cellular content from their surroundings and divide the organelles from the cytosol. Not only do they act as a barrier for the physical retention of a cell's contents, but they can also create different chemical environments on either side of the membrane. For example, cells maintain about an 80 mV electric potential across the plasma membrane and about 140 mV across the mitochondrial membrane. The ER membrane separates an oxidative environment (inside the ER) from a reduced one (in the cytosol).

The maintenance of various chemical, electrical, and redox potentials across a membrane is accomplished by various membrane proteins. Rat small intestinal enterocyte has about 150,000 Na^+ pumps per cell, which collectively allow each cell to transport about 4.5 billion Na^+ ions out of the cell each minute. The sodium and potassium membrane gradients generated by those pumps, as well as the electric potential across cytoplasmic and mitochondrial membranes, are fundamental to cellular bioenergetics.

Membrane Dynamics

The cellular membrane is in a dynamic state; membrane constituents are continuously being added and removed. This is not only for membrane expansion and cell growth, but also for turnover and vesicle trafficking. Like other cellular components, the turnover of the cellular membrane is necessary to replace lipid molecules that have been oxidized or damaged, or to allow cells to change their membrane composition to adapt to new environments. Cellular membrane proteins are also turned over (Panel 2.8).

Inter-organelle trafficking and the secretion of proteins into the extracellular environment also contribute to a membrane's dynamic state. Protein molecules that are destined for secretion or for the cell surface travel between organelles (ER and Golgi apparatus) and to the cytoplasmic membrane by residing inside membrane vesicles. Upon reaching the inner surface of the cytoplasmic membrane, those vesicles fuse with the cytoplasmic membrane and release their contents to the outside of the cell.

In the liver, each hepatocyte synthesizes ~120 x 10^3 albumin molecules per minute (translating to about 15 pg/cell/day). All of those molecules are wrapped in membrane vesicles of 280–400 nm and delivered to the basal plasma membrane of the cell. The infusion of those membrane vesicles would cause the membrane surface to expand at a rate of 0.5%/min (Panel 2.9). However, since hepatocytes are typically in a G_0 state (i.e., not dividing), the size of their cytoplasmic membrane does not need to increase to accommodate cell growth. Even if they are proliferating, the rate of membrane expansion caused by membrane vesicle fusion would be far too high for cell growth. Therefore, the lipid molecules that are added to the cytoplasmic membrane must be recycled back into the intracellular organelle (Golgi bodies) to maintain the cytoplasmic membrane in a homeostatic state.

Similarly, cells active in endocytosis can internalize up to 0.8%/min of a plasma membrane. The loss of lipids from the membrane caused by endocytosis must be replenished to maintain the size of the cell's outer envelope. Thus, cellular membranes, both cytoplasmic and organelle-related, are dynamic and maintained in a homeostatic state.

Panel 2.8. Factors Contributing to the Dynamic State of the Cellular Membrane

- Cell growth and membrane expansion
- Lipid turnover
- Inter-organelle trafficking of membrane vesicles
- Secretion, endocytosis

Panel 2.9. Homeostasis of Cellular Membranes

- Professional secretory cells in the body can add 0.5% of their plasma membrane per minute due to the fusion of secretory vesicles with the plasma membrane; they must be recycled to maintain a balance
- Phospholipids in the membrane are subject to turnover

Cytoplasm and Organelles

The cytoplasm and nucleus are both enclosed by the cytoplasmic membrane in the cell. The cytoplasm can be largely divided into two groups: organelles and the highly viscous cytosol. Cytosol has a very high concentration of proteins (100–300 mg/mL). For comparison, the protein content in blood plasma is only 90 mg/mL. Cytosol also contains the inorganic solutes, building blocks, and intermediates and metabolites of metabolic reactions.

The cytosol is not only full of soluble components (Panel 2.10). It also contains large assemblies (or aggregates) of particles. The ribosome is the main machinery for making proteins; it is a complex particle consisting of many ribosomal proteins and ribosomal RNAs (rRNA). Each cell contains thousands of ribosomes ~30 nm in size. Many ribosomes are located on the cytosolic surface of the endoplasmic membrane and appear as black spots when viewed under an electron microscope. Some enzymes also form large complexes that can be seen under an electron microscope, such as pyruvate dehydrogenase complexes.

Panel 2.10. Cytosol

- Not a simple solution
- Some protein complexes (e.g., ribosomes, pyruvate dehydrogenase) are aggregates that are visible as particles under a transmission electron microscope
- The cytoskeletal network is interspersed with cytosol

Also rich in the cytosol are the fiber-like structures of the cytoskeleton. These large protein particles, enzyme complexes, cytoskeletal proteins, and organelles make the cytoplasm of a cell very crowded and render its solution phase very dense in mass. Under light microscopy, an animal cell appears to be primarily cytoplasm wrapped in a membrane, with a nucleus sitting near the center spanning over half of the cell's diameter. Other than the nucleus, various organelles include mitochondria, the endoplasmic reticulum, the Golgi apparatus, peroxisomes, endosomes, etc., which are visible only by electron microscopy (Figure 2.6).

Nucleus

The nucleus is the largest organelle in the cell, and the only one besides the mitochondrion that has a double membrane, with both an outer and inner nuclear membrane instead of only a lipid bilayer membrane. The nucleus contains the genome, which is separated into a number of DNA molecules that each form a chromosome. Being diploid, mammals have two sets of chromosomes in each somatic cell, one of maternal and one of paternal origin. The DNA molecules are segregated into nuclear compartments. The size of a haploid genome of a rodent species of interest to bioprocessing is about 2.8 Gbp (giga (10^9) base pair). That of a human is about 3.3 Gbp. That equates to about 6–7 pg of DNA for a diploid cell. If stretched, the total DNA (from both sets of

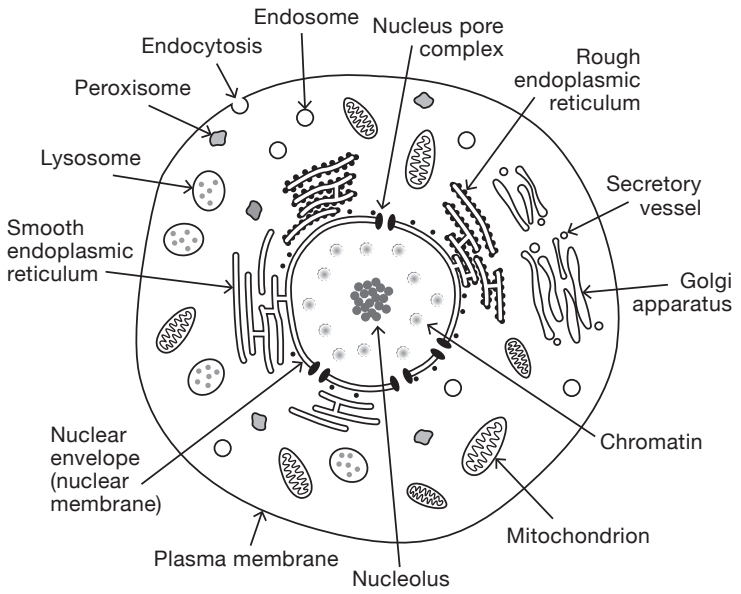

Figure 2.6. Organelles in a mammalian cell.

chromosomes) would extend to over 2 m in length. This large amount of DNA is packed into the small space of the nucleus by forming DNA-protein (histone) complexes.

The cytosol side of the outer membrane of the nucleus is associated with many ribosomes. As will be discussed later, the endoplasmic reticulum is the site of active translation. These ribosomes on the outer membrane continue to present in the cytosol side of the endoplasmic membrane surface. Much trafficking occurs between the nucleoplasm and cytosol through nuclear pore complexes that transverse both the outer and inner nuclear membrane (Panel 2.11). DNA replication, the synthesis of various RNAs (mRNA, tRNA, rRNA, non-coding (nc)RNA, snRNA, etc.), and ribosome assembly occur in the nuclear compartment. They are segregated from the metabolic processes and protein synthesis occurring in the cytosol. The nucleotides and deoxynucleotides that are synthesized in the cytosol are imported into the nucleus for RNA and DNA synthesis. Many of the RNA products, including mRNAs and tRNAs, are exported to the cytosol where translation takes place. Ribosomes are assembled from ribosomal proteins and rRNAs in nucleoli and are

Panel 2.11. Characteristics and Biochemical Roles of the Nucleus of a Human Cell

- The largest organelle in the cell
- Each cell contains ~6–7 pg DNA organized into 46 chromosomes
- When connected and totally stretched, it is ~2 m long
- Histone proteins help pack it into the nucleus
- Major reactions occurring in the nucleus:
  - DNA replication
  - RNA synthesis
  - Ribosome assembly
- Raw materials and products of these reactions are trafficked through nuclear pores

subsequently exported to the cytosol to participate in protein synthesis. Since translation occurs in cytosol, the ribosomal proteins are also synthesized in cytosol before being imported into the nucleus for ribosome assembly. The task of sorting out which segments of DNA, or which genes, are to be transcribed into RNA at a given moment occurs in the nucleus. A large array of transcription factors and other transcription regulators are also synthesized in the cytoplasm and then imported into the nucleus where they bind to specific genetic loci to perform their role in transcription.

Thus, the volume of material exchange between the nucleus and the cytoplasm is large. This large volume of traffic, from small molecules to proteins to complex protein-RNA ribosome assemblies, must be sorted so that different species move in the right direction.

Mitochondria

The mitochondrion is the organelle present in the highest numbers in a cell. With about 1,700 per cell, they make up 20% of the cell's volume (Panel 2.12). Mitochondria are about the size of bacteria and are thought to have originated from bacteria-like structures that were acquired by primitive eukaryotes.

Panel 2.12. Mitochondria

- The most abundant organelle in a cell (about 1,700 per cell)
- Use up to 20% of the cell's volume
- In the catabolism of glucose to carbon dioxide, the oxygen atom in CO_2 is contributed from water molecules. The oxygen reacts through an electron transport chain to form water with NADH and $FADH_2$ in the mitochondria.
- An active mitochondrion has a -140 mV electric potential across its inner membrane and 0.5–1.0 units of pH gradient (the pH inside the mitochondria is higher [H+ concentration is lower] and pH is pumped against the concentration gradient)
- The membrane potential cannot be increased too much. The homeostasis of mitochondria is therefore critical.
- Cells meet long-term increased energetic needs by the biogenesis of mitochondria

Mitochondria serve as the cell's power plants. The most reactive reactions in the cell (e.g., oxidizing nutrients and generating energy through electron transfer and oxidative phosphorylation) take place in the mitochondria. Cells with different energy needs have different numbers of mitochondria. In a high-energy demanding cell, there can be as many as 3,000 mitochondria.

Like the nucleus, mitochondria have a double membrane (Figure 2.7). The outer membrane maintains their macroscopic morphology, while the inner membrane is highly invaginated, often called cristae, to generate more surface area. The inner membrane is the barrier for solute transport. The outer membrane is rich in pores made of porin proteins that allow molecules smaller than 5000 daltons to pass through freely. The chemical environment of the space enclosed by the inner and outer membranes is thus largely the same as that of cytosol. The space inside the inner membrane, called the matrix, is rich in enzymes and is the place

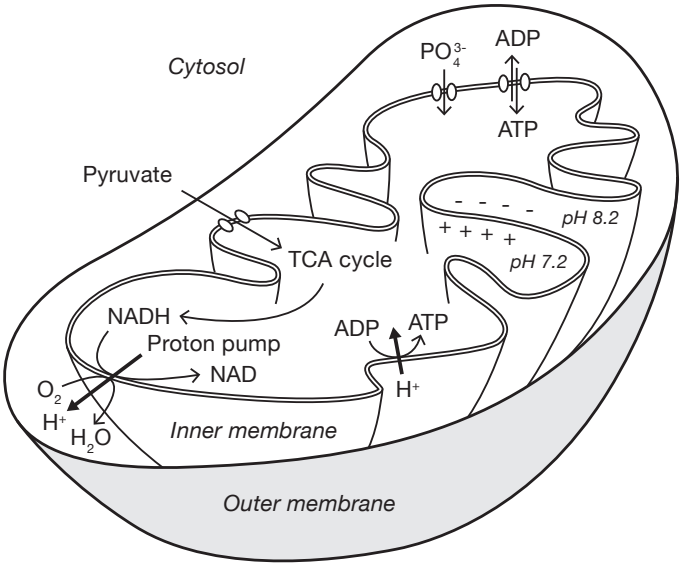

Figure 2.7. The proton and electric potential (charge) gradients, along with the material flow, cross the mitochondrial inner membrane. The direction of the fluxes of major species are indicated by arrows. Note that NADH oxidation coupled with electron transfer pumps protons against the proton and charge gradient, while the movement of protons to drive ATP synthesis is in the direction of the proton and charge gradient.

where the final breakdown of metabolic intermediates derived from glucose metabolism to CO_2 occurs. It is also where the final degradation of fatty acids and amino acids occurs when they are used to derive energy under some physiological conditions.

The chemical potential energy of pyruvate oxidation through the TCA cycle is stored in NADH. At the mitochondrial inner membrane, the electrons at a high energetic state in NADH pass down the electron transfer chain consisting of a number of protein complexes (NADH dehydrogenase, cytochrome C reductase complex, and cytochrome C oxidase complex) and pump H^+ out of the inner membrane. At the end of the electron transport, the electron reacts with oxygen to form H_2O. The ATP-generating process occurs via ATP synthase residing in the inner mitochondrial membrane. The total surface area of all mitochondrial inner membranes in a cell is greater than that of the cytoplasmic membrane. Mitochondria are thus rich in potentially damaging free radical species. The cell confines these potentially damaging reactions to the mitochondria.

The mitochondrion resembles a bacterium not only in its size but also in its possession of a genome in the form of a circular DNA molecule. Each mammalian mitochondrion contains one or more mitochondrial genomes of about 15–17 kbp that encode a number of mitochondrial proteins and RNA molecules. The control of mitochondrial DNA

replication is separate from the regulation of genomic DNA replication. The mitochondrion has its own ribosomes and tRNAs for translation, and RNA polymerase for transcription. However, many mitochondrial proteins are encoded in the cell's genome and synthesized in the cytoplasm before being imported into the mitochondria. Like other organelles, mitochondria cannot be generated from their components alone or from the genetic content in the nucleus; rather, a cell must already possess a mitochondrion in order to generate more mitochondria for its proliferation. The biogenesis (i.e., replication) of mitochondria is independent of cell division.

An active respiring mitochondrion has an about -140 mV electric potential (negative inside) and a pH difference of almost 1 unit across its inner membrane. The pH of the mitochondrial matrix is higher (i.e., the H^+ ion concentration is lower inside) than in the cytoplasm, as H^+ are pumped against the concentration gradient through the electron transport chain. The pH gradient and the electric potential are critical for the transfer of the chemical potential energy from NADH to ATP.

When the energetic need of a cell becomes high over a long period of time, it responds by increasing the number of mitochondria. Under normal (non-starvation) culture conditions, the flux of the carbon energy source (primarily pyruvate) and oxygen into the mitochondria appears to be within a narrow range. While the glucose flux into glycolysis can vary over a wide range, the pyruvate flux into mitochondria is more restricted.

Lysosomes, Peroxisomes, Autophagosomes, Endosomes

Lysosomes are small organelles where the degradation of many cellular materials takes place. Its lumen is a low pH environment (pH ~4.5) and it contains many enzymes that hydrolyze proteins, nucleic acids, and lipids (Panel 2.13). It has proton pumps in the membrane to maintain a low interior pH. It is the site of degradation of both ingested materials and cellular materials that are no longer needed by the cell. Most cellular materials have a finite life span, regardless of whether they are catalyzing chemical reactions or playing structural or mechanical roles. Over time, any cellular material can be oxidized or chemically modified in other ways in some part of its structure. The accumulation of such "damages" may render a protein non-functional. Thus, most proteins and other cellular materials, such as RNAs and lipids, are turned over after a finite period of time. Many of such processes occur in lysosomes. For some proteins, this occurs in proteasomes. Proteasomes are complexes of proteolytic enzymes that are capable of degrading proteins. Proteins that need to be turned

Panel 2.13. Lysosomes

- Low pH
- The site of degradation of endocytosed material and other cellular materials destined for degradation
- Participates in protein secretion, apoptosis

Panel 2.14. Peroxisomes

- Site of fatty acid oxidation
- Rich in oxidative enzymes

over are tagged by ubiquitin and sent to the proteasome for degradation. Lysosomes are not trash cans, but rather more like recycling centers. They play an important role in lipid homeostasis. Lipids taken up from the extracellular environment and other organelles are processed and then redistributed to maintain the proper lipid composition in the membranes of different organelles.

Panel 2.15. Endosomes

- In endocytosis, the invaginated plasma membrane forms small organelles
- Moves inward along the microtubule network
- Site of extensive cargo distribution and sorting
- Some material is sent to lysosomes
- Some is recycled to the plasma membrane

Like lysosomes and proteasomes, autophagosomes are involved in a cell's "house cleaning." However, they have double layer membranes which they wrap around the damaged materials. They can be large, as they may need to engulf damaged organelles, ingested microorganisms, or damaged proteins (a process called autophagy). They then deliver the content to a lysosome.

Through endosomes, eukaryotic cells can take up external particles by wrapping the particles within cellular membranes and taking them up as vesicles enclosed in lipid bilayers (Panel 2.14). The endocytosis pathway, discussed later in this chapter, also participates in the recycle of cellular materials involved in protein secretion. Some cells in higher organisms are specialized "scavengers" that engulf foreign particles or dead cells.

In fat cells, the catabolism of lipids occurs in peroxisomes (Panel 2.15). The reactions involved in such metabolisms generate large amounts of reactive oxygen and therefore need to be contained within these specialized organelles.

Endoplasmic Reticulum

The endoplasmic reticulum (ER) extends out of the nuclear membrane and is the organelle where the folding of proteins for secretion or delivery to many organelles and surfaces takes place (Panel 2.16). It is also a major site of lipid synthesis. It is largely classified into the smooth ER and the rough ER, based on its morphology under transmission electron microscopy. The smooth ER is rich in enzymes involved in chemical transformation reactions. In liver cells, the smooth ER takes on the role of detoxification; in the ovaries and testes, it makes hormones. The rough ER has ribosomes binding to its cytosolic surface and therefore appears rough in electron microscopy. It is the site of the synthesis, folding, and processing of proteins destined for some organelles, the plasma membrane, and secretion. It is estimated that roughly 30% of all cellular proteins are synthesized in the rough ER. Professional secretors in the body, such as pancreatic beta cells that secrete insulin and antibody-secreting plasma cells, have abundant ER. As B cells (non-antibody secreting)

Panel 2.16. The Endoplasmic Reticulum

Smooth ER

- Function varies with tissue. In liver cells it detoxifies; in ovary and testes it produces hormones.

Rough ER

- Site of folding of integral membrane proteins, proteins for some organelles, and secreted proteins
- Professional secretors in the body, such as pancreatic beta cells, hepatocytes, and antibody secreting plasma cells, all have abundant ER. As B cells differentiate to become plasma cells, the ER and Golgi apparatus expand drastically, at least by 15-fold.

Panel 2.17. Some Characteristics of the ER

- In a hepatocyte, the surface of the ER is about 63,000 μm^2 per cell, or about 40 times that of the plasma membrane
- The ER lumen is very viscous and gel-like. The diffusion coefficient of a fluorescent probe is 9–18 times lower than that for water.
- The ER has a much higher oxidative environment than the cytoplasm, appropriate for disulfide bond formation
- High free Ca^{2+} environment
- Many proteins are present at very high concentrations (PDI, GRP94, GRP74)
- A major site of protein folding, other post-translational processing, Ca^{2+} homeostasis, and cholesterol synthesis

differentiate to become plasma cells, the ER and Golgi apparatus expand drastically, more than 15-fold (Panel 2.17).

Hepatocytes secrete many proteins, including albumin, and many of the blood's protein components. In the liver, some hepatocytes specialize in protein secretion, while others play major roles in oxidative detoxification. These hepatocytes have distinctive ERs. Those involved in protein secretion have an abundance of rough ER, while those that specialize in xenobiotic metabolism have an abundance of smooth ER.

The ER is also a major site of protein post-translational modifications (Panel 2.18). The ER lumen is rich in proteins that facilitate protein folding. Disulfide bonds are formed in the ER, and the initial step of glycosylation on the asparagine residue (N-glycosylation) occurs there. The ER is involved in Ca^{2+} homeostasis, as its high Ca^{2+} serves as the reservoir to keep the cytosolic Ca^{2+} concentration low unless it is needed. The ER is also the site of lipoprotein and cholesterol synthesis.

The Golgi Apparatus and Protein Post-Translational Modification

The Golgi apparatus appears as dozens of stacks of three to seven flattened sacks in different locations throughout the cytoplasm (Panel 2.19). They are often near the ER, and the stacks are linked into a ribbon-like structure. The Golgi apparatus is loosely divided into four compartments: cis, medial, trans, and the trans-Golgi network (TGN) (Figure 2.8). The secretory and membrane proteins are transferred to the Golgi after they are folded in the ER, first to the cis Golgi and then to

Panel 2.18.
Protein Processing in the ER

- Cleavage of signal peptides
- Addition of high mannose core oligosaccharide to a Asn-x-Ser/Thr N-linked glycosylation site
- Trimming of terminal glucose and mannose residues from initial glycan
- Fatty acid addition
- Disulfide bond formation

Panel 2.19. Some Characteristics of the Golgi

- Compartmentalized into functionally distinct regions: the Golgi stack (consisting of cis, medial, and trans cisternae) and the trans-Golgi network (TGN)
- Proteins and lipids are sorted in the Golgi for delivery to different cellular locations
- From the Golgi, proteins go to secretion (exocytosis) or other organelles
- The Golgi apparatus breaks down during mitosis and reassembles afterwards

the medial and trans compartments through membrane vesicles. Then, in TGN, the proteins are sorted and transported to different organelles or to the plasma membrane for secretion. As the protein passes through the different Golgi compartments, it is chemically modified through glycosylation, sulfation, etc. (Panel 2.20). The enzymes needed for those chemical modifications are not distributed evenly in the four Golgi compartments. Thus, different reactions take place in different compartments. An estimated 100–200 glycosyltransferases, transporters of various nucleotide sugars, are the membrane proteins that constitute the majority of Golgi enzymes.

Protein Secretion through the ER and Golgi Apparatus

It has been estimated that 20–30% of all proteins encoded in a eukaryotic genome are membrane proteins that are processed through the ER and Golgi. For an industrial recombinant protein producing cell line, the secreted recombinant product constitutes a very large mass fraction of proteins passing through the ER and Golgi. These cells devote a large portion of their protein processing capacity to the secreted protein product.

Although some proteins are translocated into the ER after they are completely synthesized in the cytoplasm, most (including typical recombinant protein products) are translocated as nascent protein molecules (Figure 2.8, Panel 2.21). Proteins that are destined for secretion, the cytoplasmic membrane, and organelles have a leader sequence of five to thirty amino acids at the amino terminus. These signal sequences serve as markers of each protein's destination. After the mature mRNA with the polyadenylated tail (polyA tail) is exported to the cytosol from the nucleus,

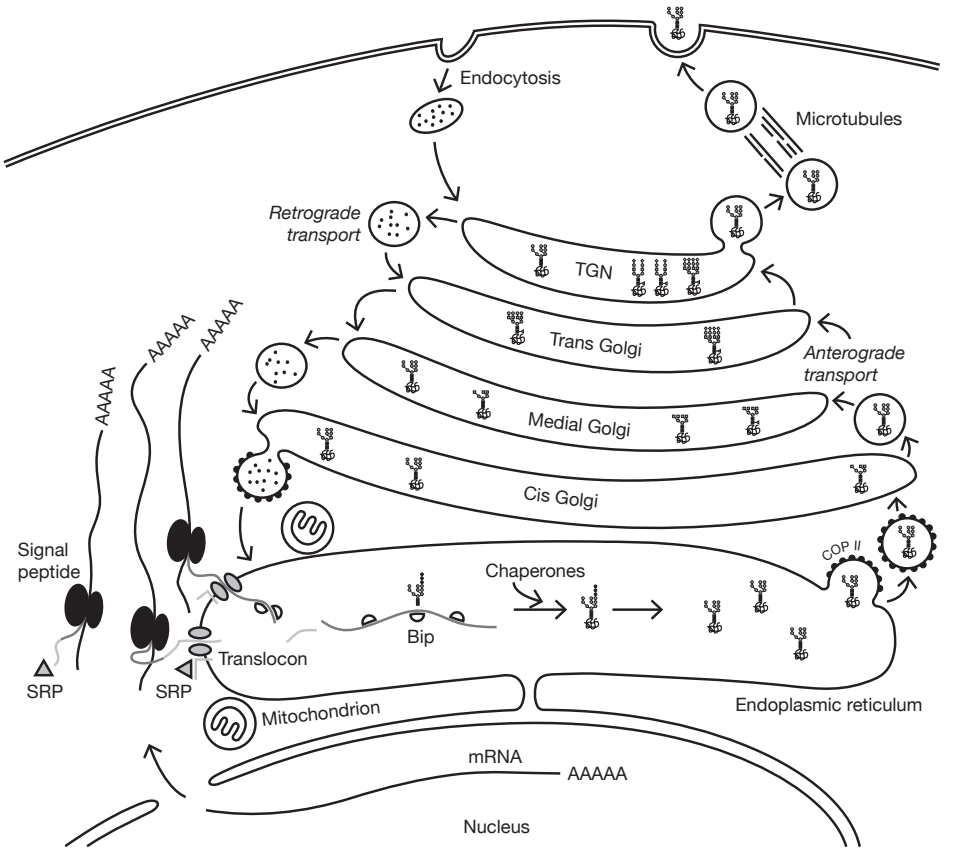

Figure 2.8. Synthesis and secretion of proteins to the extracellular environment. See Panel 2.21 and text for details.

Panel 2.20. Protein Processing in the Golgi

- Addition glycoform modification
- Sulfation of tyrosines or carbohydrates
- Glycosylation
- Peptide proteolytic cleavage
- γ-carboxylation of glutamic acid
- β-hydroxylation of aspartic acid

a ribosome binds to the translation start site of the mRNA and starts translation. The signal peptide of the nascent protein is synthesized and then recognized by signal recognition particles (SRP) near the ER. The binding of SRP to the signal sequence causes a pause in translation and docks the nascent protein (which has only the beginning segment of the entire sequence) along with the ribosome and the mRNA to a translocator (also called a translocon or translocation channel) on the ER membrane. The nascent polypeptide is then transferred through the translocator into the ER lumen, but the ribosome and mRNA are left on the cytosolic surface of the ER. Subsequently, translation elongation resumes and the elongating polypeptide passes through the channel of the translocator into the ER lumen. As the translation proceeds downstream of the mRNA,

new ribosomes continue to bind to the translation initiation site of the mRNA, initiating the synthesis of the signal peptide and repeating the process of translocating the nascent protein into the ER. Thus, on the cytoplasmic side of the ER each mRNA molecule has multiple ribosomes actively translating the protein next to the translocator.

Folding of the polypeptide starts immediately upon translocation into the ER lumen. The signal peptide on the elongating polypeptide in the ER lumen is cleaved upon entry into the ER. A class of ER chaperones and other proteins that facilitate protein folding act on the nascent protein molecules to prevent them from aggregating and to facilitate folding. Their actions require cellular energy (ATP). An important member of the ER luminal chaperones, BiP (also known as GRP78), is also a component of the translocator complex. BiP binds to hydrophobic amino acids in incompletely folded proteins to prevent them from aggregating. In addition to BiP, major ER luminal chaperones include protein disulfide isomerase (PDI), which facilitates the formation of the correct disulfide bond.

Throughout the process of protein secretion, N-glycosylation takes place on the protein, starting in the ER and continuing into the Golgi (Figure 2.8, also Figure 3.21). It is called N-glycosylation because the oligoglycan is linked to the asparagine residue of specific sequences on the protein. As the protein is being synthesized and folded, a preassembled oligoglycan containing three terminal glucose residues is transferred and covalently linked to the asparagine (Figure 2.9). The addition of glycan to the protein increases its stability in a soluble form. It is also an important marker of protein folding. While the protein undergoes folding, the glucose residues are cleaved. A glucosyl transferase adds a glucose to the aglycosylated glycan on any protein that

Panel 2.21. Protein Secretion

- Nascent protein molecules destined for the ER have a special ER signal sequence being synthesized in an organized polysome. They are recognized by SRP (signal recognition particle), a ribonucleoprotein.
- SRP binding transiently arrests elongation, directing the ribosome/nascent polypeptide complex (RNC) to a receptor on the ER membrane and transferring the growing polypeptide to a translocon
- SRP is released from the RNC
- The nascent polypeptide begins to pass through the translocon and elongate into the ER lumen
- Signal peptide on the elongating polypeptide is cleaved
- Protein folding and post-translation modification begins as the polypeptide continues to elongate
- Major ER luminal chaperons: BiP, calnexin, calreticulin, and PDI
- The ribosome is released once the translation is complete
- Folded proteins (with an inner core of glycan if it is a glycoprotein) concentrate at the exit site of the ER and are thought to bud into vesicles and translocate to the Golgi as pre-Golgi intermediates
- The Golgi apparatus is in a dynamic state. Transport is retrograde (recycling its own proteins) and anterograde.
- After reaching the trans Golgi network (TGN), secretory proteins are packaged into post-Golgi vesicles and moved along the cytoskeletal network through the cytoplasm to fuse with plasma membrane and be secreted

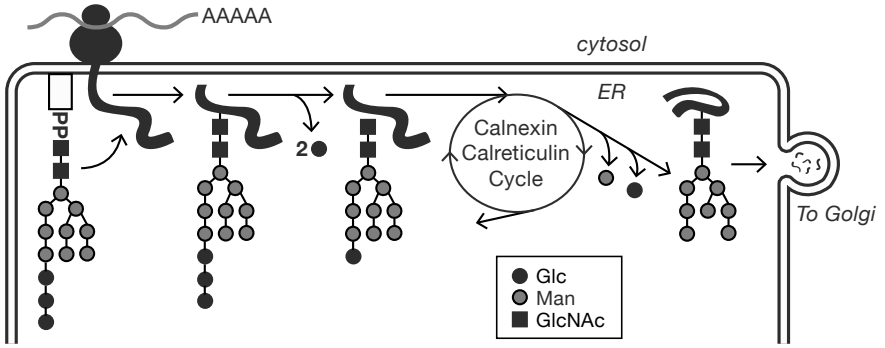

Figure 2.9. Glucose trimming from an N-glycan as a signal of correct protein folding.

is not correctly folded, leaving the completely folded protein with an N-glycan that does not have any terminal glucose. Calnexin and calreticulin are lectins (glycan-binding proteins). They bind to the glycan on the protein that has only one glucose residue and retain them in the ER for further folding. Once the protein is completely folded and has no terminal glucose, it is ready for transfer to the Golgi apparatus. The protein molecules that are improperly folded are then exported to the cytosol and degraded in a proteasome.

Protein molecules that have completed the folding process are localized in some regions of the ER where the ER membrane is coated with a protein COPII. Membrane vesicles that contain the folded proteins and are coated with COPII are then budded out from the ER and exported to the cis-Golgi. In the Golgi, further post-translational modifications take place. The cis-, medial-, trans-Golgi, and TGN comprise an array of tubules which are tethered as microbioreactors in a series, and in which different enzymes carry out different modifications on the protein. The medial-Golgi, typically containing three to seven stacks of cisternae, is the main site of glycan elongation for glycoproteins. Vesicle budding from a compartment and fission with another are the main forms of protein trafficking from the ER to the Golgi and among Golgi compartments. The surface of the Golgi compartment is decorated with proteins (golgin) that form coiled-coils that act almost like tentacles to help capture the vesicle.

In addition to secretion, proteins are also bound for different organelles and different regions of the plasma membrane. In TGN, proteins are sorted into membrane vesicles according to their destination. The vesicles are coated with different proteins to mark their destination. The transport of the membrane vesicle is facilitated by microtubules. As will be discussed later, microtubules can polymerize and depolymerize quickly to become track-like, and as such can be used to guide the vesicle cargos to the right location.

As the trafficking happens, the membrane and the contents of an early compartment are translocated to a later compartment of the ER, the Golgi, different organelles, and the plasma membrane. Such a material flow needs to be balanced by a similar material flow in the opposite direction. Thus, the protein secretion process involves not only anterograde transfer of membrane vesicles to the later compartments and plasma membrane, but also endocytosis and retrograde transport to return the membrane and other constituents to their originating compartment in order to maintain each compartment at a homeostatic state. The ER, Golgi, lysosomes, and endosomes are thus all part of the secretory network. They communicate through the dynamic trafficking of membrane vesicles. After the translation is completed, a finite amount of time is required for a protein molecule to be folded, glycosylated, and then excreted. The complete translation of an average protein about 350 amino acids in length takes only about a minute. In comparison, the secretion of a synthesized protein molecule takes from tens of minutes to a couple of hours depending on the nature of the protein. The α1-protease inhibitor is among the fastest secreted proteins, with a half-life of about 28 min (Table 2.3). The secretion of transferrin and IgG takes about two hours. In general, a secreted protein molecule spends more time in the ER than in the Golgi apparatus.

One can view the process of protein cargos traveling through different compartments towards the TGN as a production stream passing through a series of bioreactors. There are two idealized scenarios on how the protein molecules pass through, one like a plug flow reactor (PFR), the other a continuous stirred tank reactor (CSTR). These two reactor types will be discussed in Chapter 8. In a PFR model, all molecules move along like a marching band in a parade, with those entering the reactor at the same time exiting it together. In a CSTR model, the molecules enter the reactor and then run around in a circle inside, with each molecule having an equal chance of being randomly taken out regardless of how long it has been in the reactor. If the protein secretion process in the Golgi apparatus behaves like a PRF, all molecules will be subjected to the same degree of enzymatic modifications; if it behaves like a CSTR or a number of CSTRs in a series, some will spend more time in the reactor and

Table 2.3.
Secretion Times of Liver Proteins

Protein	*Half-life in ER (min)*	*Half-life in Golgi (min)*
Transferrin	110	45
Ceruloplasmin	80	30
Anti-trypsin	30–40	10
IgG	Total ~120	

Panel 2.22. Processing Time of Secreted Proteins

- Translation of a protein molecule takes only seconds, but the secretion process takes tens of minutes to hours
- Different proteins spend different amounts of time in the ER for folding, and in the Golgi apparatus for other post-translational modifications
- Different molecules of the same protein have different holding times in the ER and Golgi. The half-life of a protein in the ER and Golgi is the average of all molecules.

possibly be modified by enzymes more extensively than others. The nature of protein secretion is likely to be neither a PFR nor a CSTR. Nevertheless, the holding time of a secreted protein is likely to distribute over a range.

Experimental observation has indeed shown that the secretion time of different molecules of the same protein is not uniform, but instead distributes over a range (Panel 2.22). This was illustrated by arresting protein secretion using a drug and then removing that drug; upon resumption of protein secretion, the fluorescently labeled protein molecules travelled through different cellular compartments at different rates. The glycosylation reactions in the Golgi, from a core oligoglycan to the completely extended product, involve more than ten enzymes distributed over different compartments. Since different molecules spend different amounts of time in each compartment, they are subject to varying degrees of different reactions. This distribution of residence time in the Golgi contributes to the heterogeneity of glycosylation.

Transport across Cellular Membranes

The lipid bilayer membrane that separates the cell from its environment, and an organelle from cytosol, presents a barrier to material exchange across it. It has a very low permeability for large and small molecules alike (Figure 2.10). Among the nutrients and metabolites of cells, only oxygen, fatty acids, ethanol, and some lipids can freely pass through the membrane fast enough to meet the needs of cell growth. Specialized transport mechanisms are needed to mediate the movement of the vast majority of nutrients and the excretion of metabolites.

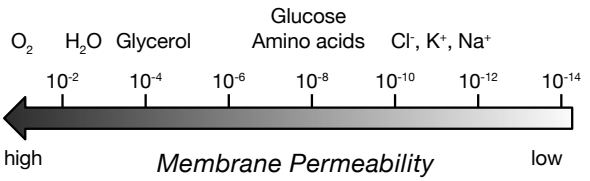

Figure 2.10. Orders of magnitude of the permeability of some solutes across a lipid membrane.

Cells have a large number of transporters (sometimes called permeases) that allow small molecules (up to about 1 kDa, e.g., sugar, oligosaccharides, amino acids, oligopeptides, nucleotides, cholesterol, ions, organic acids, etc.) to cross the cytoplasmic membrane and the membranes of various organelles. Macromolecules are transported across membranes by membrane vesicles (as in the secretion process through the ER and Golgi apparatus), pinocytosis, or exocytosis. Specific receptors are involved in taking up large proteins and complexes such as low-density lipoprotein (LDL) and transferrin.

Types of Solute Transporters

Active and passive transport

The cellular transport of solutes across a membrane can be classified into two types: passive transport, which transports a solute along its concentration gradient, from the high concentration side of the membrane to the low concentration side, and active transport, which passes the solute from the low concentration side to the high concentration side (Figure 2.11, Panel 2.23). The former is thermodynamically favorable, whereas the latter must be coupled to an exergonic process to make the transportation possible. One such coupled process that provides the chemical potential energy necessary for active transport is the hydrolysis of ATP. Another is the co-transfer of a second solute along a large concentration gradient across the membrane. The chemical potential energy change of the co-transport of the second solute provides a sufficient net energy change to drive the transport of the first solute against its concentration gradient. For example, the intestinal sodium-dependent glucose transporter relies on the more than 10-fold higher concentration of sodium in the exterior to drive glucose to move from a low-level area in the gut lumen to the interior of intestinal cells with a higher glucose level. Thus, the driving force of sodium transfer "pushes" glucose to move against its concentration gradient.

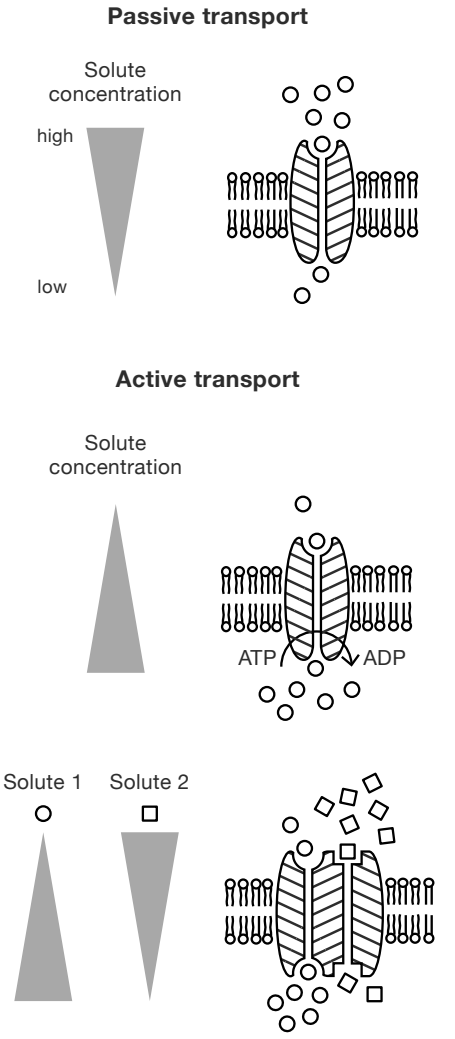

Figure 2.11. Active transport and passive transport. Active transport requires the supply of chemical potential energy derived from ATP hydrolysis or co-transport of a solute such as H^+ or Na^+.

Transporter and channel proteins

From a structural perspective, the transport of a solute along the concentration gradient can be mediated by a carrier protein (transporter) or by a channel protein (Figure 2.12, Panel 2.24).

Channel proteins open into a duct-like structure across the membrane that is specific to a particular solute, such as water, Na^+, or K^+. Channel proteins exist in either an open or closed state. Once the channel is open, the transfer of solutes is very fast in the direction of decreasing concentration. The flux is very

Panel 2.23. Types of Solute Transport

Active Transport

- Moves molecules against their concentration gradient
- Requires ATP or ion gradients (H^+, Na^+) as an energy source to drive transportation

Passive Transport

- Moves chemical species from the high concentration side to the low concentration side of a membrane
- May involve channel proteins or facilitated diffusion transport

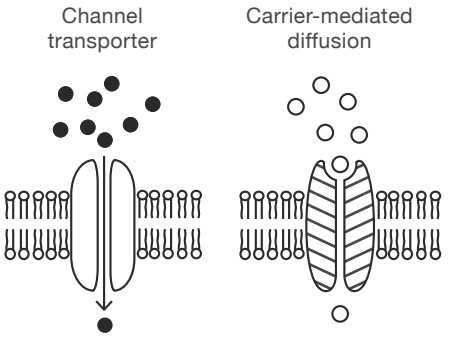

Figure 2.12. Channel proteins and transporter proteins.

Panel 2.24. Three Classes of Transport Processes

Channel-Protein-Mediated Diffusion

- Molecules or ion-specific
- Once a channel is open, very fast flux

Transporter-Facilitated Diffusion

- Provides specific bonding to a solute
- Solute binding causes protein conformation change to allow for transfer to the other side of the transporter
- Opening the transporter allows the release of the solute to the other side of the membrane

fast and is affected by the number of channel protein molecules on the membrane and by the time period that the channel is open.

Carrier-mediated transporters mediate facilitated diffusion. First, the solute from the high concentration side of the membrane is diffused into the transporter and docks to the binding domain of the transporter. Next, the solute is translocated to the low concentration side of the membrane. Once on the low concentration side, the solute is exposed and is free to diffuse away. Under normal culture conditions, glucose and some amino acids are transported by facilitated diffusion.

Transporters for facilitated diffusion mediate reversible transport. The rate of transport by a transporter is dependent on the concentration difference of the solute across the membrane. Typically, the transporter has a high affinity for the solute on the donating side, which helps facilitate the binding of the solute, and a lower affinity on the downstream side to facilitate the release of the solute. If the concentration of the solute on one side is much greater than the other, the rate generally depends only on the solute level in the donating side. The mechanism of such transport is similar to a typical enzyme-catalyzed conversion of a substrate to a product. The dependence of the transport rate to the solute concentration can be described by a Michaelis–Menten kinetic equation. At low concentrations, the transport rate is in proportion to the solute concentration. At high concentrations, the rate is constant as the transporter becomes saturated. A half-saturation constant (K_M) is used in the kinetic expression of the transport rate as a function of solute concentration. K_M is the solute concentration when the transport rate is half of its maximum.

A ubiquitous transporter for glucose is the glucose transporter 1 (GLUT1). The half-saturation constant (K_M) for GLUT1 is

about 0.1–1 mM. In the 0.1–1 mM range, the glucose import rate of the cell increases with increasing glucose concentration. In the concentration range often used in cell culture media (1–10 g/L, or 5.5 mM to 55 mM), the glucose transport rate is near its maximum and is not affected by glucose concentration.

Uniporters and co-transporters

Transporters for facilitated diffusion and active transport can also be categorized according to the number of solutes each carries and the direction of solute flow (Figure 2.13, Panel 2.25).

Uniporters transfer a single solute from a high concentration side to a low concentration side, e.g., the GLUT1 transporter for glucose and the GLUT5 transporter for fructose. Symporters and antiporters transfer two solutes simultaneously. A transporter that delivers the two solutes in the same direction is called symporter. Conversely, an antiporter transfers two solutes in opposite directions.

Collectively, symporters and antiporters are called co-transporters. Co-transporters are often used to transport charged organic molecules. Dissociable solutes move with a counter ion to maintain electric charge neutrality. When a charged solute moves from one side of the membrane to the other, the charge neutrality must be maintained. Otherwise, a net charge will accumulate across the membrane and create an electric potential that will retard further transfer of the solute. For example, lactic acid exists as lactate in an aqueous solution at neutral pH. If lactate moves from one side of a membrane to the other without being accompanied by a positively charged species such as H^+, the cell membrane will become negatively charged on the lactate-receiving side and positively charged

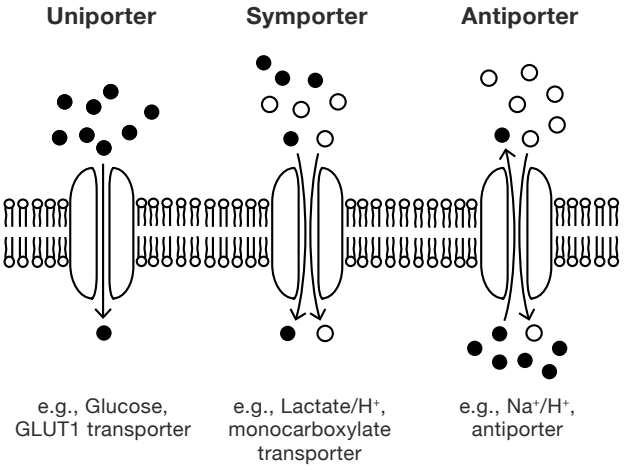

Figure 2.13. A uniporter and the two types of co-transporter: symporter and antiporter.

Panel 2.25. Transporters

Uniporter
- Transfers a single molecule (e.g., glucose, fructose)
- The solute is mostly uncharged

Bispecies Transporter (Co-Transporter)
- Requires a stoichiometric exchange of two species simultaneously
- Important in charge balance
  - Symporter
    - Two species transported in the same direction
  - Antiporter
    - Two species transported in opposite directions

on the lactate-donating side, eventually creating a voltage across the membrane that prevents further transport of the negatively charged lactate. Thus, many dissociated organic acids are transported by a co-transporter so that a charge does not build up across the membrane.

Two mechanisms are commonly employed to maintain a charge neutrality across a membrane: a) co-transport with a counter ion (such as H^+ for lactate) by a symporter, and b) co-transport with an ion of the same positive or negative charge, but in the opposite direction (such as Cl^- for HCO_3^-).

In co-transporter mediated transfer, the transport rate is not only affected by the concentration difference of the solute across the membrane, but also by the concentration gradient of the co-transported ion. For example, the transport rate of lactate by the monocarboxylate transporter (MCT) is not only affected by the concentration of lactate, but also by the pH difference between the cytosol and the medium.

"Pump"-like transporters

We use pumps to transport water. Sometimes a pump is used to overcome gravity and move water higher, and other times the water is going downhill anyway, but the pump is used to move it faster. In solute transport across a membrane, energy is sometimes used to overcome the concentration gradient (active transport) such as in the case of pumping water uphill; in other cases, energy is used to make the transport faster (Figure 2.14).

The Na^+/glucose transporter (SGLT) in the epithelium of the intestine can take up glucose from the digestive track even when glucose levels are

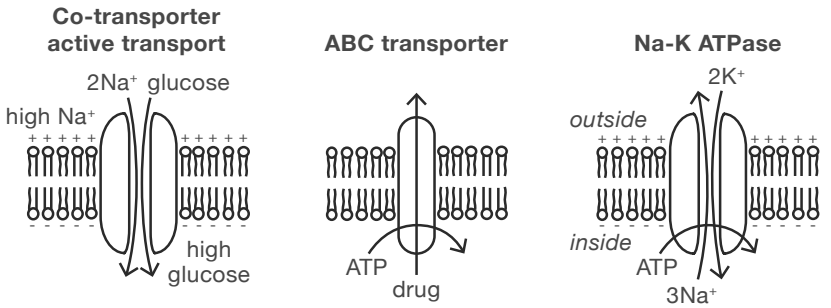

Figure 2.14. "Pump"-like transporters.

lower than in the cell. This is accomplished by using the Na^+/glucose transporter to transport two Na^+ from the lumen of the intestine into the cell, where the Na^+ level is very low (Figure 2.14). As the sodium ion is transported, a glucose molecule also binds to the transporter and is transported simultaneously. The propensity of Na^+ to move across the membrane is so high that it can drive glucose to move against a large concentration gradient. The driving force for Na^+ to cross the membrane is not only from the concentration difference of Na^+, but also from the attracting power of the negative membrane potential, the –80 mV across the membrane.

Another type of pump-like transporter for active transport is the ATP-binding cassette (ABC) transporter, which transports some hydrophobic compounds by utilizing ATP as the energy source (Figure 2.14). Its mutation is involved in many human diseases. It is also involved in the development of resistance to many drugs by pumping the drug out of the cell, causing a low intracellular concentration of the drug. Even if its efflux is along its concentration gradient, the transport rate will be low. With an ATP-driven transporter, the rate is enhanced. After prolonged exposure to methotrexate, some cancer cells can develop drug resistance by pumping the drug out of the cells using ABC transporters, thereby becoming drug-resistant. A third type of pump-like transporter, Na-K ATPase, will be described below.

Ion transport and membrane potential

The intracellular fluid contains a variety of inorganic ions. Some trace elements (Fe^{3+}, Zn^{2+}, Co^{3+}, Se^{2+}) are present only at very low concentrations. Many ion species (Na^+, K^+, PO_4^{3-}, Mg^{2+}, HCO_3^-, Cl^-) are present in the millimolar range (Panel 2.26). All rely on transporters to cross the cell membrane. Some are also transported by channel proteins in a regulated fashion for controlling membrane potential and maintaining its homeostatic level in the cell. Those ions are present in the cytoplasm in very different concentrations. They also face varying concentration differences across the cell membrane. For instance, Na^+ and K^+ have opposite directions of concentration gradient across the plasma membrane. The intracellular concentration of K^+ is 20–50 times higher inside than outside of the cell, while the Na^+ concentration is 10–15 times higher outside the cell versus inside.

Along with the concentration gradients of major ions, cells also maintain an electric potential gradient of –80 mV across their plasma membrane (negative inside the cell). This electric potential is fundamental to the transport of many compounds across the membrane. Because of the large capacitance in the lipid bilayer membrane, it takes the transfer of only an extremely small number of positively charged ions (e.g., K^+, Na^+) from the cytoplasm to the extracellular space to create the 80 mV charge difference. The number of ions transferred to generate

Panel 2.26. Transporters for Ions

- The concentrations of many major ion species (H^+, Na^+, K^+, Ca^{2+}, PO_4^{3-}, Cl^-) across membranes are polarized. Na^+ and K^+ have opposite directions of concentration gradients across the plasma membrane, about a 10-fold difference. For Ca^{2+}, the intracellular concentration is so low that the gradient is extremely steep.
- Na^+-K^+ ATPases play a major role in maintaining Na^+ and K^+ gradients. ATPase utilizes the hydrolysis of ATP as its energy source.
- Iron is extremely reactive and participates in many redox reactions. In biological systems, it exists in its "bound" form to binding proteins or chelators. In its free form, it catalyses the formation of peroxide and peroxidizes unsaturated fatty acids.

the membrane potential is many orders of magnitude lower than the total number of ions in the cell, and their transfer does not cause any change in their intracellular concentration.

Sodium-potassium ATPase plays a key role in maintaining the intracellular levels of Na^+ and K^+ (Figure 2.14). It transfers 2 K^+ into the cytoplasm and 3 Na^+ out of the cytoplasm using ATP as the energy source. It is present in the cytoplasmic membranes of all mammalian cells. The cytoplasmic membrane is not absolutely impermeable to ions. It allows Na^+ and K^+ to diffuse across at a very slow rate. Due to their small difference in the membrane permeability, K^+ diffuses faster than Na^+. Intracellular K^+ also leaks out through potassium channels. The combined actions of Na^+/K^+ ATPase and leakage through channels and the membrane facilitate the balance of the Na^+ and K^+ gradient and the membrane potential. Cells also have chloride ion pumps to pump negatively charged Cl^- out of the cell. In some cells, Na^+ and Cl^- channel proteins also facilitate the maintenance of the membrane electric potential in the correct range. The electric potential across the membrane plays an important role in solute transport, as illustrated by the Na^+/glucose transporter.

Cell Shape, Mechanics, and Movement

Cells present themselves in many different shapes. Those circulating in blood are mostly spherical. Many epithelial cells are cubical, while fibroblast cells grown on Petri dishes spread out like a sunny-side-up fried egg, complete with elongated spindles. The shape of a cultured cell tells a lot about the cell type, the status of its differentiation, and its health. Before modern biochemical and molecular biological analytical tools were available, the observation of cell shape was, and still is, a primary tool for judging the "state" of a cell. The shape of a cell is the result of the force balance among the cell's internal structural organization and the interactions between the cell and adjacent cells as well as between the cell and the surface that it attaches to. Cell shape is thus a reflection of a cell's mechanical properties.

Cell shape is dynamic. Cells move from one place to another for various reasons. In embryo development, they may move to a new site

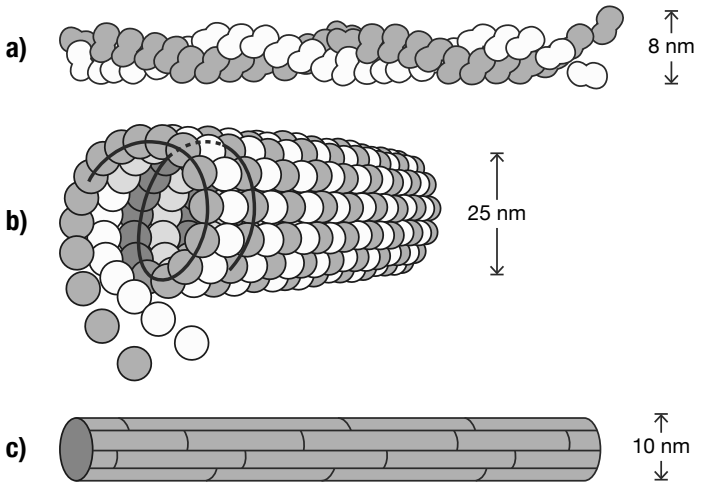

Figure 2.15. (a) An actin filament, (b) a microtubule, and (c) an intermediate filament.

to differentiate into a new tissue. In an adult, they may move to repair a damaged tissue. Two daughter cells move away from each other after cell division. As a cell moves or divides, its mechanical properties, shape, and interactions with other cells and the surface also change. In short, a cell's internal mechanical structure, interactions with adjacent cells, and interactions with the surface it attaches to together influence its shape, movement, and intracellular as well as extracellular mechanical actions.

A cell's internal mechanical structure is governed by the proteins of the cytoskeleton. Cell–cell interactions are influenced by junction proteins and a number of cell–cell adhesion molecules. Cells secrete extracellular matrices that overlay a surface with characteristics that facilitate the differentiation of itself or other cells. These extracellular matrices are also subject to dynamic modifications during their development.

These aspects of cell culture used to be of lesser interest to process engineers when the focus was solely on highly adapted recombinant cells growing in suspension. With increasing interest in cells for regenerative medicine and cell therapy applications, maintaining a cell's differentiation status is critical and a better understanding of cell–cell and cell–surface interactions is important.

Cytoskeleton

Three major components make up the cytoskeleton of a cell: actin filaments, microtubules, and intermediate filaments. These give cells their shapes and transmit forces within them (Figure 2.15). Both actin filaments and microtubules are assemblies of their respective monomers of globular proteins (actin and tubulin, respectively). The assembly of monomers requires chemical potential energy in the form of ATP (for actin) or GTP (for tubulin). The stacking of monomers into the assembly

is done without forming covalent bonds among monomers. Intermediate filaments are also polymers but are made of monomeric filament proteins. Actin filaments, microtubules, and intermediate filaments are all helical-type fibers with multiple strands that can reach substantial lengths. Actin filaments, at about 8 nm in diameter, are the smallest of the three. Microtubules are hollow, with a diameter of about 25 nm. Intermediate filaments have a diameter of about 10 nm.

Actin filaments and microtubules can quickly grow or shrink in length by assembling or disassembling monomers. Because monomers form the assembly in a head-to-tail fashion, their filaments are polarized. The filaments and the monomer interact with a number of auxiliary proteins that modulate the filament into a dynamic or static state and determine the network type that the filaments form. The monomer proteins also contain a nucleic acid (ATP in actin, GTP in tubulin) that is converted to ADP or GDP in the assembling process.

Panel 2.27. Actin Filaments

- Two-stranded filaments (F-actin) of helical polymer of actin (G actin) (50 kD)
- 5–9 nm flexible structures organized into linear bundles, 2-D networks, and 3-D gels
- In non-motile cells, actin filaments form bundles called stress fibers, creating a loose mesh of filaments that underlie the cell membrane
- Distributed all over the cell, but concentrated in the cortex beneath the plasma membrane
- Projections from cells such as microvilli, lamellipodia, microspikes, and filopodia are maintained by rigid bundles of actin filaments
- In actively moving cells, stress fibers disappear and actin filaments concentrate at the leading edge.
- There are many actin-related proteins which affect the polymerization and motor functions of actin filaments
- Actin is involved in motor functions such as muscle contraction

Actin filaments

Actin filaments are comprised of two or three strands that, like rope, are twisted to form a single-string filament (Figure 2.15, Panel 2.27). This geometry enhances their structural integrity while maintaining a high degree of flexibility. A large number of auxiliary proteins provide control of the nucleation of actin monomers and direct their assembly into different types of networks with varying degrees of actin filament length, cross linking, bundling, and tethering to the cell membrane. These auxiliary proteins help regulate the spatial distribution of different types of actin filament networks in the cell.

A high concentration of actin is often seen immediately underneath the cell's plasma membrane, forming a gel-like structure called the cortex. In immunofluorescent microscopy, following the staining of cells with anti-actin antibodies, actin appears in web-like bundles. The cortex acts as the first absorber of external mechanical perturbations and gives the lipid bilayer its local shape.

Many cell types, such as fibroblasts, extend their body and spread flat on a surface, both in tissues and in culture. The edge of an adherent cell has an irregular shape, much like an egg laid on a pan. In the protruding regions, actin fibers

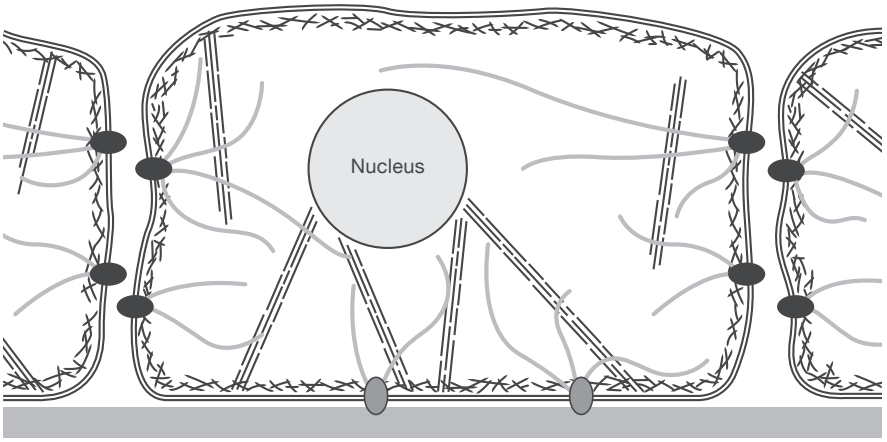

Figure 2.16. Cytoskeleton of a cultured fibroblast cell in culture in an epithelial cell layer. Actin filaments are shown as intersecting hash marks, intermediate filaments as fluid gray lines, and microtubules as dashed compound lines.

localize in the lamellipodia and filopodia. In the protruding filopodia, actin filaments form intensely labeled parallel bundles of fibers. While in the lamellipodia, they form cross-linked non-parallel networks. The two types of network can reorganize, the filopodium region protruding out from a lamellipodium during cell movement. In stationary cells, the actin fibers form long stress fibers that may span a large fraction of the entire cell length. The stress fibers connect to the focal adhesion (the location where the cytoplasmic membrane is in contact with the substrate surface) and establish a tension force between the cell and the extracellular matrix. Stress fibers also connect to adjacent cells through adhesion junctions.

Microtubules

Microtubules are hollow tubes formed by assembling 13 threads of α- and β-tubulins (called protofilaments) together (Figure 2.15 and Figure 2.16, Panel 2.28). Like actin filaments, the head-to-tail assembly of the monomers renders a microtubule directional, giving rise to a "+" and a "–" end. The two ends of the microtubules can extend or shrink quickly by assembling or disassembling reactions. The negative ends of many microtubules converge in an area called a microtubule organizing center (MTOC). The largest MTOC is the centrosome, from which a large number of microtubules extend their "+" ends outward. Many auxiliary proteins bind to or interact with microtubules and tubulins. A number of microtubule-associate proteins (MAPs) stabilize microtubules or facilitate the interactions of microtubules to cell components, such as binding to the cell membrane. Other auxiliary proteins promote disassembly, nucleation, bundling, or cross-linking. Microtubules are thus major

Panel 2.28. Microtubules

- Long, hollow tubes of polymerized subunit tubulin (MW 50 kD), about 25 nm in diameter, more rigid than actin filaments
- Typically long and straight, many have one end (–) attached to a single microtubule organizing center (e.g., centrosome)
- α- and β-tubuline (GTP) forms heterodimers; the dimer assembles in a head-to-tail fashion into chains called protofilaments, 13 of which make up the microtubule wall
- Can polymerize to extend and depolymerize to retract its length rapidly
- Play a key role in intracellular organelles and small vesicle transport
- For secretory proteins, the movement of post-Golgi vesicles to the plasma membrane is mediated by microtubules

Panel 2.29. Intermediate Filaments

- Play a structural role by stabilizing and transmitting mechanical force
- The subunit is not a globular protein, but fibril, different from tubulin and actin
- 10 nm diameter, has a head, a tail, and an α-helical rod; can be made of a wide variety of proteins in various tissues

structural components that affect cell shape, both statically and dynamically.

Microtubules are also the major player in a cell's motor functions. They accomplish this by serving as the track that motor proteins, like kinesin and dyneins, can slide along with expenditure of ATP. The motor protein, by forming complexes with other proteins that bind to various cargos, such as excretory membrane vesicles or organelles, can then transport the cargo along the microtubule.

Intermediate filaments

Unlike actin filaments and microtubules, which are ubiquitous to all eukaryotic cells, intermediate filaments are only present in some animals, including vertebrates, nematodes, and mollusks (Figure 2.15 and Figure 2.16, Panel 2.29). Although all intermediate filaments share common structural features, they are comprised of many different molecules and their expression is tissue specific. For example, laminins are present in the nucleus, vimentins in many mesenchymal cells, and keratins in epithelial cells. The major role of intermediate filaments is to transmit mechanical force and provide cells with their mechanical characteristics. For example, the intermediate filament keratin gives the outer layer of skin its toughness. While the diverse intermediate filaments are not conserved in their amino acid sequence, they are conserved in major protein domains and share common characteristics in their molecular organization. A common feature of intermediate filaments is that they form a head-to-tail coiled heterodimer, and then a pair of dimers form an antiparallel and symmetrical tetramer. Thus, intermediate filaments fundamentally differ from actin fibers and microtubules in that the latter two are polar with a "+" and a "–" end. Tetramer subunits are stacked together to form a filament. Each intermediate filament fiber is made of multiple fibrils, which are in turn made of a series of subunit proteins.

These intermediate filament fibers are flexible. Unlike microtubules and actin fibers, which can have tens of micrometers of persistence length, intermediate filaments can bend within a short distance and are capable of absorbing energy exerted by external forces and transmitting

it to other regions of the cell. In a tissue or in interconnected cells in culture, intermediate filaments also help to transmit forces between cells.

Extracellular Matrices and Integrins

The vast majority of cells in the body are embedded in an acellular tissue structure composed of proteins and polysaccharides (Figure 2.16, Panel 2.30). In tissue differentiation, these structures may become calcified bone, cartilage, the connective tissue underlying the epithelium layer, or other specialized tissues, or form the basement membrane (also known as basal lamina) that cells sit on. A variety of glycoproteins (proteins linked with oligosaccharides) and proteoglycans (a core protein with one or more glycosaminoglycan (GAG)) secreted by various cells provide the core components of ECM (Panel 2.31). For example, osteoblasts secrete collagens, other bone-forming proteins, and hydroxyapatite; chondroblasts secrete collagen, hyaluronic acid, and proteoglycans; and fibroblasts secrete collagens and fibronectin. The tissue cells not only secrete different ECM components, but also organize them into different structures to give the special characteristics of different tissues. For example, collagen fibers may be aligned in one direction rather than being randomly coiled.

Panel 2.30. Extracellular Matrix

- The substrate for adhesion of many cell types
- Provides cues for growth, differentiation, and development
- Rich in electrocharges, allowing growth factors, cytokines, etc., to be "stored" inside them

The chemical composition as well as the physical organization of ECM as a cell's local environment affects the differentiation and maintenance of differentiation properties. When cultured *in vitro* on plastic or glass surfaces, cells secrete those materials onto the surface after adhering to form the ECM on which they reside. For *in vitro* culture of embryonic stem cells, laminin-coated culture dishes are used to maintain their pluripotency; while in culturing primary hepatocytes isolated from liver, type I collagen is used. Many ECM components are highly negatively charged. This allows many protein growth factors to be adsorbed to the ECM and released to surrounding cells, perhaps even serving as a chemoattractant. Therefore, they also play a role in providing cues for cell migration and differentiation.

Panel 2.31. Components of the ECM

ECM Proteins
- Collagen
- Laminin
- Fibronectin

Proteoglycan
- Chondroitin sulfate

Glycosaminoglycan
- Heparin
- Hyaluronic acid

Integrins and cell–ECM interactions

The biological role of ECM is not merely to provide a surface appropriate for cell adhesion, but also to provide cell-surface interactions that are linked to the expression of special cell characteristics and growth control (Panel 2.32, Figure 2.17). Cells of the same type may exhibit very different shape and other cell behavior when cultured on different ECMs. The different responses to different ECMs is mediated by

Panel 2.32. Integrin

- Provides mechanical and signaling interactions between the intracellular and extracellular environments
- Can sense extracellular ECM components and transmit signals to the cytoskeleton
- Consists of different α and β chains for heterodimers of different integrins

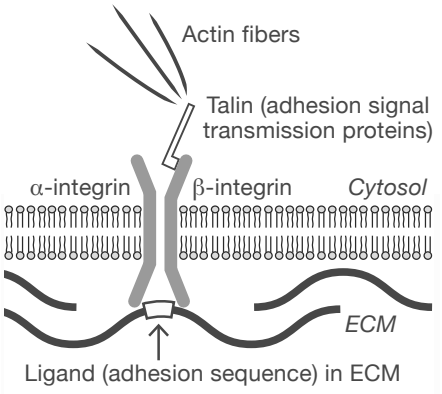

Figure 2.17. Integrin and interactions between ECM and the cytoskeleton.

transmembrane cell surface receptors, called integrins, that connect the extracellular ECM to the intracellular cytoskeleton and relay the chemical and physical features of the extracellular microenvironment to intracellular signaling events. The integrins are heterodimers of different α and β chains. Different combinations of α and β chains gives the specificity of binding to different ECM molecules. For example, $\alpha_5\beta_1$ integrin binds to fibronectin, while $\alpha_6\beta_1$ binds to laminin. On the intracellular side, integrin binds mostly to actin filaments with the help of some auxiliary binding proteins. Different cell types express different α and β chains and thus form different integrin complexes with different binding affinities for various ECM components. As a result, different cell types often have different ECM requirements for adhesion, growth, or maintaining their differentiated properties. To establish strong and stable interactions between ECM and the cytoskeleton, multiple cell–matrix junction complexes cluster together to form focal adhesion.

The integrin heterodimer can exist in either an active state that engages ECM, actin filaments, and other associated proteins, or in an inactive state. This switch of state allows cells to exert or disengage internal and external mechanical interaction. The switch of state is facilitated by coupling to a cell's signaling proteins. The cell–matrix junction is associated with signaling kinases or other signaling system proteins. The signal transmission occurs from both sides of the cellular membrane, from intracellular to extracellular environment and vice versa. The binding of integrin to ECM proteins can activate integrin to allow for binding by actin filaments. Conversely, internal signaling activation of integrin can also initiate the connection of actin filaments and ECM through integrin.

The inward pulling of actin filaments through a cell–ECM junction results in a tension force that is transmitted through cytoskeletal fibers. In the case of anchorage-dependent cells, the tension force provides the necessary signal to allow the cell cycle to proceed.

Cell Movement

The vast majority of cells are capable of movement on surfaces. In general, cell movement can be the result of an attraction to diffusible chemicals or favorable ECM on the surface, or random movement. Cell movement involves a restructuring of the cytoskeleton, a protrusion of

the membrane, the establishment of surface adhesions on one side of the cell, and finally the detachment of the cell membrane from the adhesion complexes in the rear end of the cell. The actin fibers and plasma membranes of moving cells extend the cell into a more elongated shape and form lamellipodia that may also contain microspikes called filopodia (Figure 2.18, Panel 2.33). The reorganization of the actin fibers happens within a few minutes in the leading edge of a moving cell.

On an "open" surface (i.e., uncrowded), cells move randomly. They exhibit locomotion and contact avoidance, meaning that, when two moving cells encounter each other, both will move away in opposite directions. Furthermore, the two daughter cells of a dividing mother cell move away from each other when cell division is complete. Cell migration is regulated by many factors, including growth factors. For example, epithelial cells respond to hepatocyte growth factor by moving away from each other and becoming more scattered, instead of forming the cell clusters typical of epithelial cells.

Cell migration is not intentionally controlled or manipulated in cell bioprocessing. In the case that adherent cells are plated on a surface for cultivation, they may redistribute themselves after cell plating. However, since their movement is largely random, their ability to distribute themselves through contact avoidance is limited to a local region of tens of cell length. It is still important to distribute them uniformly during cell inoculation.

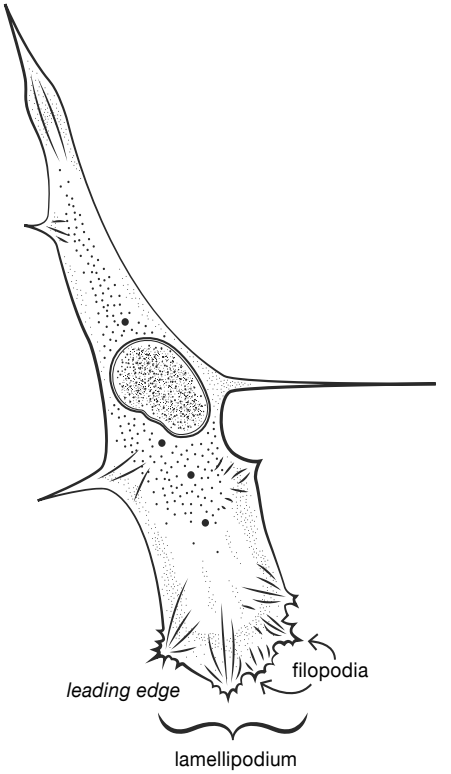

Figure 2.18. A fibroblast in locomotion. Lamellipodia and a few filopodia extend from the leading edge.

Panel 2.33. Cell Movement

- Filopodia extend as a result of actin fibers growing at the cell front, and establish a "grip" on the surface
- The subsequent dissociation of cell–substrate contact at the rear of the cell allows the cell's center of mass to move forward

Growth Control

The growth of an animal from embryo to adult is regulated at cellular levels in different tissues through the control of the increase in both cell mass and cell number. The increase in cell mass and cell number may not always be synchronous, but over the course of a few cell generations they occur simultaneously. Although we see only the growth aspect of the development, at a cellular level the growth and sustenance of an

Panel 2.34. Growth Control

Growth is the Balance of

- Positive factors: promote increasing cell mass/cell division, suppress cell death
  - Availability of nutrients, growth factors, mechanical tension through the cytoskeleton and surface adhesion
- Negative factors: promote programmed cell death, suppress growth
  - Sensing presence of death signal, apoptosis-triggering stress

organism involves not only cell growth, but also cell death. Both the growth and death of a cell are tightly regulated, or programmed. The most well-studied programmed cell death is apoptosis. Cell growth control and apoptosis will be the focal points of the following discussion.

The growth of a cell is the manifestation of a delicate balance between positive and negative regulations that respond to signals both from outside and inside the cell (Panel 2.34). Positive regulations stimulate cell growth and proliferation and suppress cell death mechanisms, while negative regulations suppress growth and promote cell death. External signals from the environment tell cells the availability or absence of nutrients and other factors necessary for DNA replication and biomass synthesis. Meanwhile, internal signals assess the cell's structural integrity and physiological readiness for cell division and modulate cellular programs to increase cellular component content, divide, or die. Apoptosis is regulated in a similar way to growth control, by pro- and anti-apoptotic factors that respond to both internal signals of cell damage and external signals of developmental cues.

Cell Cycle and Growth Control

Eukaryotic cells progress cyclically through four stages in their undertaking to grow in number: G_1, S, G_2, and M phases (Figure 2.19, Panel 2.35). G_1 and G_2 refer to the gap phase. S and M phases derive their designation from DNA synthesis and mitosis, respectively. The four stages constitute a cell cycle which is repeated every time a single cell becomes two daughter cells. In G_1 phase, cells increase in mass and cell size. Its duration is variable depending on the chemical and physical environment of the culture. The cell will also check whether the internal and external conditions are ready for proceeding to the next phase of the cell cycle, S phase. A restriction point is considered to exist before the entry into S phase. Cells pass the restriction point and enter the S phase only if cellular conditions are right, the necessary external positive mitogenic factor is available, and the negative inhibitory factor is absent. For example, PDGF (platelet derived growth factor) is required for the proliferation of fibroblasts, EGF (epidermal growth factor) for epithelial and many non-epithelial

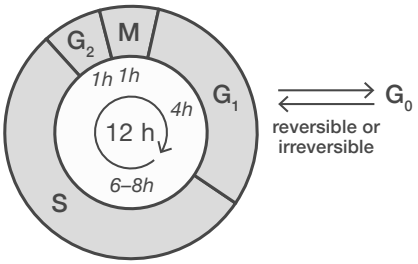

Figure 2.19. The cell cycle for a mammalian cell.

cells, and EPO for red blood cell precursors. The entry into S phase for anchorage-dependent cells additionally requires established contact between integrin and ECM and the appropriate tension in the cytoskeletal network. Countering the actions of the positive factors are those factors that provide signals to cause growth arrest. For example, TGFβ (transforming growth factor-β) inhibits the proliferation of many cell types. Cell-cell contact after anchorage-dependent cells have grown to a confluent state causes growth to cease. For cells that require the establishment of cell–cell contact for growth, the dissociation of adherent junctions between cells disrupts the cells' internal signaling networks and causes growth arrest. The process of mitosis and cytokinesis takes about an hour for a mammalian cell, while the S phase takes around 6 hours. While the doubling time (i.e., a period of cell cycle) changes under different culture conditions, G_1 phase is the stage that is extended most.

Panel 2.35. The Cell Cycle

- Proliferative cells go through the G_1, S, G_2, and M phases of the cell cycle
- Quiescent cells exit G_1 and enter G_0
  - G_0 can be reversible and the differentiated cells returned to the G_1 phase (e.g., hepatocytes), or irreversible for terminally differentiated cells (e.g., nerve cells)
  - Major professional secretory cells in the human body (hepatocytes, plasma and pancreatic cells) are at G_0 phase
- The progression of the cell cycle is regulated by different cyclins, Cdks, and CDI inhibitors, each of which is dynamically expressed
- Cell cycle control is linked to programmed cell death control through factors that affect both

Cells that are in a long period of quiescence, such as terminally differentiated cells, divert from G_1 to exit the cell cycle and enter G_0 stage. G_0 stage may exist in two different types: a resting state that allows cells to eventually reenter the cell cycle and proliferate, or a state that has permanently inactivated the cell cycle reentry mechanism. For example, terminally differentiated cells such as neuronal cells in the body are at a permanent G_0 stage. Fibroblasts in the body and in culture that are under amino acid starvation conditions enter a resting G_0 stage. They can reenter G_1 phase and resume growth under appropriate conditions.

During S phase, DNA replication takes place. DNA replication in the somatic cells of an organism has a very high fidelity, at the sequence level as well as the macroscopic structural level of chromosome. All $>10^{12}$ cells in our body have basically the same genome sequence and chromosomal organization. At the end of S phase, a cell has duplicated its genome or DNA content in the correct sequence and with an extremely low error rate. This may not be the case for some cancer cells or cell lines in culture. For example, at DNA sequence levels, segments of genomic DNA in CHO cells can be seen to have gained or lost copies compared to the rest of the genome. When CHO cells, which are known for having an aneuploid karyotype, are derived from a clone, they originally have the same chromosomal organization. After a few generations, however, their offspring have very different numbers of chromosomes and composition.

Following G_2 phase, the replicated genome, in the form of two sets of chromosomes, segregates in the M phase (called cytokinesis). As the nucleus divides into two, each of them receives one set of the chromosomes that constitutes a diploid genome. The cytoplasmic materials and nuclei are then distributed to two daughter cells. The process of cytokinesis has a very high fidelity. However, this is not the case in continuous cell lines and cancer cells. Errors in DNA synthesis and repair in those abnormal cells give rise to abnormal chromosomes, and mistakes in cytokinesis result in a heterogeneous population where different cells have different makeups of chromosomes (i.e., abnormal karyotypes). Such lack of fidelity in genome replication control is part of the nature of those cells. After isolating a single cell to start a new cell population, the chromosome makeup of the population becomes heterogenous again, even though the entire population was from a single ancestor karyotype.

Cyclins and CDKs

The progression through the cell cycle (G_1, S, G_2, and M) is positively regulated by cyclins and cyclin-dependent kinases (CDKs) and negatively controlled by CDK inhibitors (CDIs). Stage-specific cyclins for the G_1, G_1/S, S, and M phases rise and fall during the cell cycle and interact with different CDKs to form cell cycle stage-specific Cyclin-Cdk complexes. The different Cyclin-Cdk complexes are further regulated at activity levels by activating and inactivating phosphorylation and by complexing with CDI. Additionally, Cyclin-Cdk complexes are subject to proteolytic regulation (Figure 2.20).

Each of these regulatory proteins displays a characteristic dynamic profile throughout the cell cycle (Figure 2.21). The activity profile of the Cyclin-Cdk complex is the result of the expression and interactions of its components, which are further subject to activation, inactivation, and degradation, all choreographed to a rhythm. Importantly, the choreography is dynamic, able to respond to environmental cues such as nutrient abundance or starvation, DNA damage, or chromosomal segregation disorder.

An important cell cycle checkpoint occurs during the transition from G_1 to S phase. The pivotal players in the G_1/S phase transition are the regulatory retinoblastoma protein (Rb) and the Cdk4/6-Cyclin D complex (i.e., G_1-Cdk complex) (Figure 2.20). In growth-arrested or quiescent cells, Rb is unphosphorylated and binds to and inhibits its E2F. E2F is a transcription factor that activates the transcription of Cyclin E and other proteins for S phase. When in a Rb bound state, it suppresses their transcription. Upon stimulation of mitogen and other cues, ERK (a pivotal player of the growth regulatory signaling system) activates the G_1-Cdk complex (via a transcription factor like c-Myc) to phosphorylate Rb. The phosphorylated Rb dissociates from E2F, leading to the activation

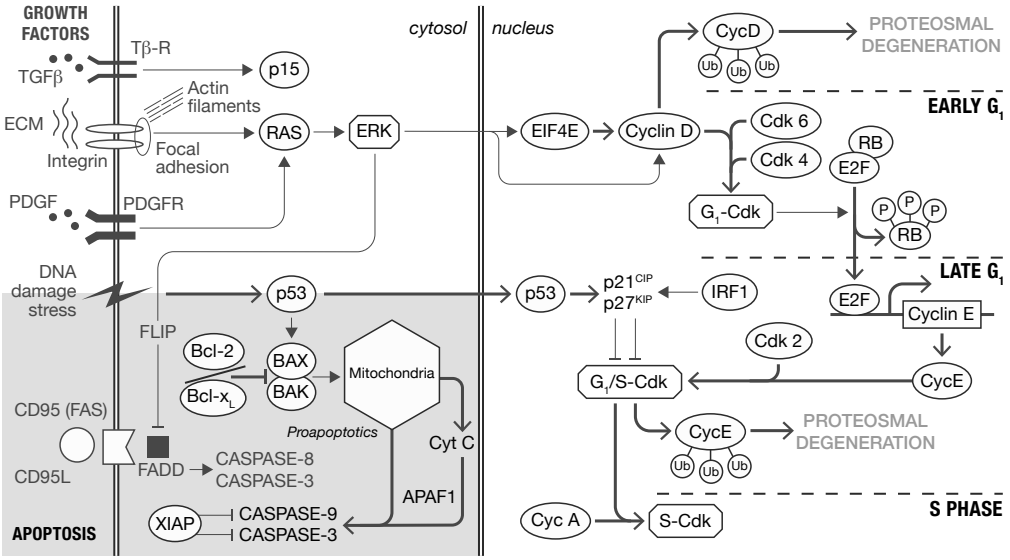

Figure 2.20. Schematic representation of the interactions between cell growth regulation and apoptosis regulation. Cdk: cell cycle-dependent kinase, IRF1: interferon regulatory factor 1, RB: retinoblastoma, ERK: extracellular signal-regulated kinase, FADD: FAS-associated death domain protein, FLIP: FLICE-inhibitory protein, EIF4E: eukaryotic translation initiation factor 4E, Cyc: cyclin, XIAP: cross-linked inhibitor of apoptosis proteins, APAF1: apoptotic peptidase activating factor 1, BAX: Bcl-2 associated X protein, BAK: Bcl-2 homologous antagonist/killer, Ub: ubiquitination, Cyt C: cytochrome C.

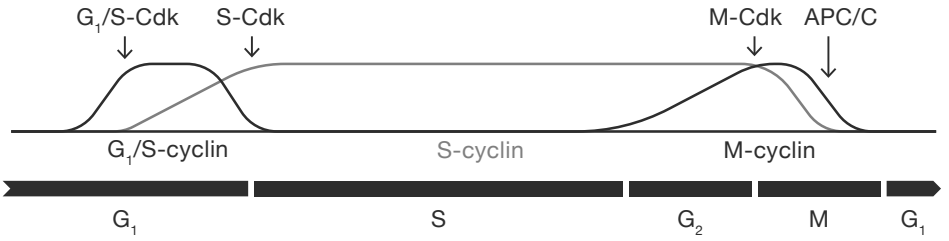

Figure 2.21. Dynamic expression of cyclins in different phases of the cell cycle.

of expression of Cyclin E that in turn drives Cdk2 activation and the formation of cyclin E-Cdk2 (G_1/S Cdk complex). E2F positive feedback on the phosphorylation of Rb causes hyperphosphorylation of Rb and activation of Cdk2. The S phase cyclin, Cyclin A, begins to accumulate in late G_1 phase, but is bound by an inhibitor. The G_1-Cdk complex facilitates the removal of the inhibition to enable the accumulation of S-Cdk complexes, marking the entry into S phase.

The requirements of internal and external conditions for passing the restriction point are strictly followed in normal cells, but not in cancer cells and continuous cell lines. Virtually all cell lines used for the

production of therapeutic proteins, including CHO, BHK, HEK293, and mouse myeloma cells, have lost their normal growth control. Their cell cycle checkpoint controls have been compromised and the requirements of mitogens and cell-adhesion complex formations have been relaxed. However, the molecular mechanisms responsible for growth control relaxation are not completely characterized in every cell line. CHO cells have an amplified genomic region that encodes for c-Myc gene, an oncogene, and a mutated TP53, a tumor suppressor gene. The product of gene TP53, p53, mediates external stress signals to cell cycle regulators to constrain the cell cycle. Its genome is also highly structurally abnormal in terms of DNA duplication and deletion and chromosomal reorganization. What additional genomic alterations exist in the cell cycle control in CHO cells that give them their highly erratic growth behavior (e.g., growth factor- and anchorage-independence) is still not clear.

Programmed Cell Death and Apoptosis

Most cell death in an organism is part of a regulated or programmed process, rather than simply the result of extensive damages. Apoptosis is the process of regulated cell death in response to developmental cues or to accumulating non-lethal stresses (Panel 2.36). There are many reasons a cell might be programmed to die for the benefit of the organism. In some developmental events, more cells are generated than will eventually constitute a differentiated tissue, and the excess ones will undergo apoptosis and die. The dead cells are subsequently ingested by neighboring cells and disappear. This is seen in neuronal tissue development, where nerve cells are produced in large numbers. The cells require positive survival signals that are limited in number. Those which receive a sufficient quantity of positive signals survive to constitute the neuronal tissue, while those that do not undergo apoptosis and die out. Some cells are generated to serve their function only for a set period of time. After they outlive their intended active time span, they are programmed to die. For example, immune cells, like antibody-producing plasma cells and natural killer cells, serve their functions for a period of time and then enter apoptosis and die. Cells that have been

Panel 2.36.
Programmed Cell Death and Apoptosis

- Most cell death is programmed
- Purposeful cell death
  - Developmental need, provides space for other cells to grow
  - No longer needed in the organism
  - Infected by a pathogen or otherwise damaging to the organism (e.g., cancerous)
- Programmed cell death includes apoptosis and necroptosis
- Two types of apoptosis:
  - Extrinsic pathway: mediated by death receptor
  - Intrinsic pathway: stress signal causes cytochromes and proapoptotic components in the mitochondria to release, activating proapoptotic proteins and triggering caspases to degrade cellular components
- Caspases-mediated cell destruction exhibits characteristic DNA fragmentation, nuclei condensation, and blebbing. *In vivo*, the dead cell is phagocytosed by other cells.

infected by pathogens and damaged also enter programmed cell death, thus reducing the spread of the pathogen to other uninfected cells.

The types of programmed cell death are traditionally grouped by the morphological changes of dying cells, although the molecular mechanisms have become better known in recent years. Apoptosis is marked by specific cell morphological changes: mitochondrial membrane rupture, DNA condensation and fragmentation, chromatin shrinkage, and membrane bulging. The final intracellular event involves a series of cascades leading to cellular destruction. *In vivo*, the dead cell and debris are often engulfed by neighboring phagocytotic cells before their complete rupture. The second type of cell death, necroptosis, is similar to necrosis, i.e., cell death caused by accumulation of damages. It is marked by the rupture of the cytoplasmic membrane and the release of the cell's contents, thus possibly contributing to inflammatory responses such as seen in the programmed cell death of immune cells.

Apoptosis associated with developmental events is mediated by the binding of extracellular ligands to the death receptor on the cell membrane, called the extrinsic pathway. The other pathway, the intrinsic pathway, is activated under cell stresses, such as nutrient depletion, growth factor deprivation, virus infection, or metabolite accumulation. The intrinsic pathway is also referred to as the mitochondrial pathway. The stress induces an intracellular signal that activates the intrinsic pathway and thus causes cell death.

The final acts of self-destruction through both types of pathways are similar in all apoptosis mechanisms. However, the initiating "signal" of the death receptor pathway and the mitochondrial pathway are different (Figure 2.20).

Extrinsic pathway

In many developmental events, individual cells serve their function for only a finite period of time. These cells are programmed to enter apoptosis after their functional life span is reached. Their survival is dependent on the presence of positive factors and the absence of negative effectors. Developmentally related apoptosis is largely regulated by death receptors on the cell surface. The death receptor pathway is mediated by the binding of the ligand to death receptor.

Cells subject to developmentally regulated apoptosis express death receptors, such as the Fas death receptor, on their surface. The binding of an external Fas ligand to the death receptor recruits an adaptor molecule, Fas-associated death domain (FADD), to the cytoplasmic end of the receptor. The presence of FADD causes pro-caspase 8 or 9 to associate with the death receptor, forming a death-inducing signaling complex (DISC). The caspase is then proteolytically activated, triggering the activation of a series of downstream effector caspases (3, 6, and 7). The activation of

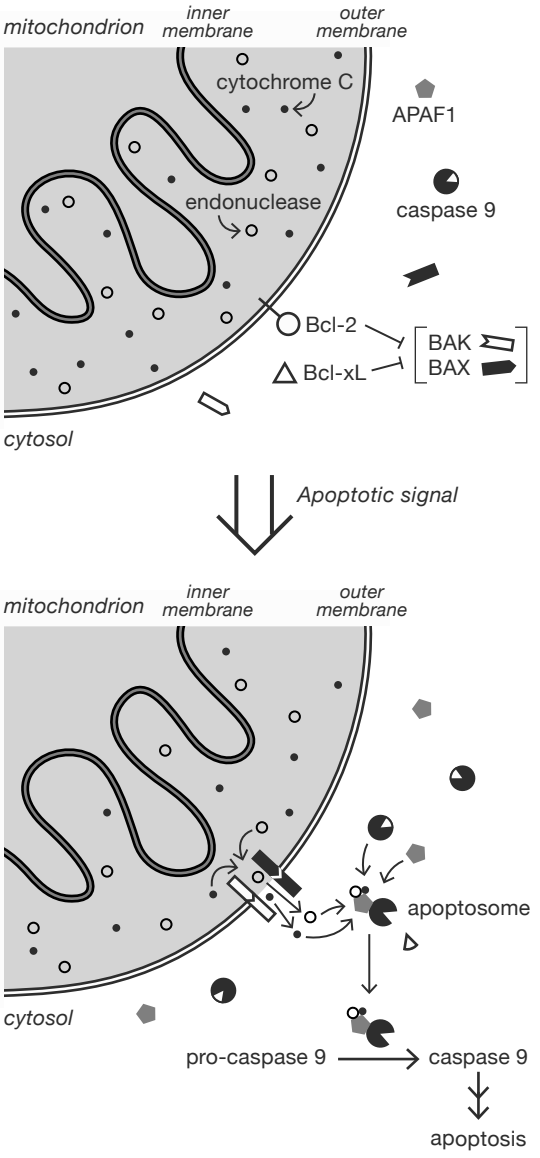

Figure 2.22. An intrinsic (mitochondrial) pathway of apoptosis.

these effector caspases leads to the final stages of cell destruction.

Intrinsic (mitochondrial) pathway

In addition to their role in energy metabolism, mitochondria also play a key role in the regulation of apoptosis. Some pro-apoptotic proteins are sequestered in the space between the outer and inner membranes of mitochondria. Cytochrome C, a hemeprotein that is an important component of the cytochrome C complex in the electron transport chain, is associated with the inner membrane of mitochondria. The release of cytochrome C and those pro-apoptotic proteins in stressed cells initiates the intrinsic pathway of apoptosis (Figure 2.22). The cytochrome C released into the cytoplasm proceeds to form a complex with APF1, pro-caspase 9, and dATP, known collectively as the apoptosome. In the apoptosome, the inactive pro-caspase 9 becomes activated and subsequently activates downstream caspases.

The mitochondrial apoptosis pathway involves positive pro-apoptotic and negative anti-apoptotic factors. The Bcl-2 family that consists of over 20 pro- or anti-apoptotic proteins is a major player in the mitochondrial apoptosis pathway. The pro-apoptotic subfamily includes BAX, BAK, and BOK, which all contain BH1, 2, and 3 homology domains.

Upon exposure to death signals, BAX undergoes conformational changes and translocates to the mitochondria, where it inserts itself into the outer mitochondrial membrane and forms channels. These channels allow the leakage of cytochrome C and other pro-apoptotic molecules.

Two anti-apoptotic proteins, Bcl-2 and Bcl-xL, counter the actions of the pro-apoptotic components. Bcl-2 is localized on the mitochondrial membrane and inhibits the release of pro-apoptotic molecules from the mitochondria by maintaining membrane integrity. Bcl-xL is localized

in the cytoplasm and binds to pro-apoptosis members of the Bcl-2 family. The involvement of multiple protagonistic and antagonistic factors ensures tight control of the apoptotic event.

For cells in culture, apoptosis also occurs under some stress conditions. It is tempting to delay apoptosis and prolong the duration of cell cultivation by using chemical means or genetic modification. For example, a large number of caspase inhibitors have been explored for use *in vitro* as well as in animal models. Overexpression of anti-apoptotic genes in cultured cells has been shown to delay the decline of cell viability in the late stages of fed-batch culture.

Concluding Remarks

This chapter has provided an abbreviated overview of the key biological features of cells that are essential for biotechnologists to practice cell culture process. The structure and makeup of cells gives them their functional versatility but also constrains their capability. While exploiting their biological versatility, we must also understand the cells' structural and functional constraints and biological limits. We must keep in mind that the objective of cell bioprocessing is to fully harness cells' biological potential for technological applications. For that, the limit may not be bounded by a cell's nature; rather, engineering can be used to facilitate the fulfillment of our goal. By equipping ourselves with a better knowledge of cells' capabilities and limits, we will be able to push the technological boundary further.

References

1. Freshney, I., *Biology of cultured cells*, in *Culture of animal cells: a manual of basic technique and specialized applications*. 2010, John Wiley & Sons, Inc. p. 11.
2. Alberts, B., Johnson, A., Lewis, J., Morgan D., Raff M., Roberts, K., and Walter, P. *Molecular biology of the cell (6th Edition)*. 2015, Garland Science.