2

Proliferation and Death of Animal Cells: Molecular Mechanism

Proliferation and death are two opposing phenomena in all living organisms. The synthesis of nucleic acids, proteins, carbohydrates, lipids, and other biological molecules accomplish growth at the cellular level. Following a period of growth, cells reproduce by division. In the pattern of growth and division that increases body cell numbers in mammals, the daughter cells are exact genetic duplication of the parent cell. In mammals, unwanted cells are removed during *embryogenesis*, *metamorphosis*, and tissue turnover by the process of appoptosis. Similarly, the proliferation and death phenomenon can be observed in cultured cells. Despite being outside the body, each mammalian cell responds to a stimulant almost similarly as it is believed to respond *in vivo*. The events for the growth of mammalian cells have been described in this chapter, which primarily include cell cycle, molecular basis of proliferation, growth factor signaling, etc. Apoptotic cell death, its induction in cell culture and possible mechanisms of apoptotic death are also included here.

2.1 CELL GROWTH

In multicellular organisms, growth normally refers to an increase in size and/or weight. However, in an isolated cell (derived from a multicellular organism), growth implies proliferation, which means the increase in cell number. It is usual that the individual cell also increases in cell mass before it undergoes a division. In mammals, about 200 different specialized cell types are present. Despite complicated patterns of interdependency, individual cell is responsible for its own maintenance, and in principle cells grow and divide independently of their neighbors. An adult human has about 10^{14} cells derived from single cell (fertilized oocyte), indicating extensive multiplication potential of animal cells. As exceptions,

event in each phase is completed before moving to the next. For that the *checkpoints* are strategically placed in the late G_1 phase and at the G_2/M phase, to monitor the integrity of DNA, in order to prevent progression and propagation of mutated or damaged cells. G_0 phase refers to cells that are quiescent (temporarily or permanently out of cycle). The normal quiescent cell is dependent on external stimuli to exit the G_0 phase and enter the cell cycle through the early part of G_1 phase.

Mitosis is essential for growth and division of cells. It is a dramatic, coordinated change in the architecture of the cell that segregates the replicated chromosomes into two identical sets and initiates the cell division (Figure 2.1C). The entire set of duplicated DNA is segregated prior to the division, giving each cell 23 pairs of chromosomes. Mitosis is divided into four phases: 1) **Prophase:** chromosomes condense into visible structures, spindle fibers begin to extend

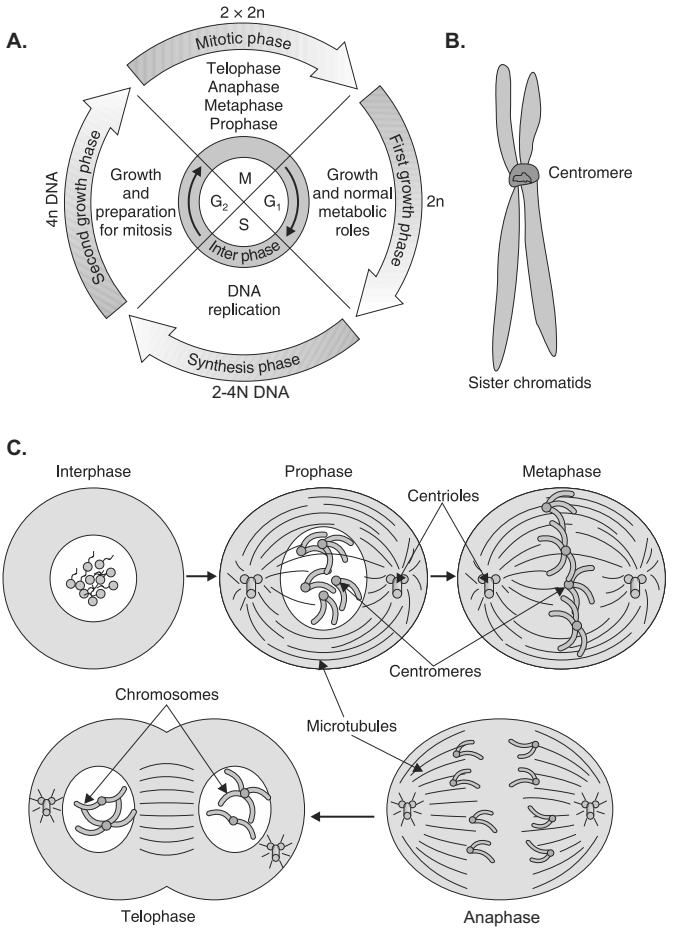

Figure 2.1 Diagrammatic presentation of different cellular events during the division of animal cell. **A.** Cell cycle; **B.** Sister chromatids; **C.** Different phases of mitosis in somatic cells.

from *centrioles*, *centrosomes* move to opposite poles of cell and the process ends with the disappearance of nuclear membrane. 2) **Metaphase:** each pair of sister chromatids (joined by a common centromere) attach to microtubules originating from opposite poles of the spindle and align along the equatorial plane of the cell (midway between the two spindle poles). 3) **Anaphase:** centromeres split, freeing the sister chromatids (now called daughter chromosomes) that move along the microtubules to opposite poles of the spindle. 4) **Telophase:** immediately after anaphase, the physical process of cell division, called *cytokinesis* begins when a contractile ring pinches the cell into two daughter cells, each containing a complete set of chromosomes and a spindle pole, the chromosomes decondense and acquire a nuclear envelope, reforming an interphase nucleus, and the microtubule array returns to its interphase pattern, and finally cell splits into two daughter cells.

In mammals, the length of cell cycle may vary from cell to cell. In general, cells do not differ much in the duration of S, G_2, and M phases, which vary between 2-6 h, 2-4 h, and 1-2 h, respectively. The longest phase in the cell cycle is the G_1 phase, which may vary between 12 to 96 h, depending on the type of cell. The embryonic cell cycle is in general much shorter than somatic cell cycle, because G_1 and G_2 phases are largely suppressed during early development of embryos. It is known that, transformed cells grow more rapidly than normal somatic cells, both in culture (*in vitro*) as well as in the body. It is important to mention here that G_0 phase does not exist in transformed cells, and therefore mitotic signals are not required for them to grow. On the other hand, in absence of mitotic signals, somatic cells remain in G_0 phase. Somatic cells may also undergo *differentiation* or *apoptosis* in the absence of mitotic signals.

2.1.2 Study on cell cycle

Biochemical studies on cell cycle require a population of cells that proceed synchronously through the cell cycle, which means that cells progress in the same phase of the cell cycle. However, somatic (adult cells) and transformed cells are not synchronous. The DNA content of a cell reveals its position in the cell cycle. The study of cell cycle not only explains the position of cells in the cycle, but also assists to determine the *generation time* and the *mitotic index* of the particular cell type, and the presence of *apoptotic* cells and *heterokaryons*, if any. The cells in S phase can be detected by their ability to incorporate labeled DNA precursors whose presence can be detected in cell lysate. To determine the length of cell cycle, the cells are labeled with high specific activity [^{3}H]-thymidine (5 μCi/ml, 2 Ci/mmol) for 30 min. The unincorporated isotope is washed-off, and the appearance of the labeled cells (mitotic) is determined *autoradiographically* at 30 min intervals for a period of 48 h (Maciera-Coelho, 1973). In theory, the first labeled mitotic cell will appear after a time equal to the length of G_2 phase and the percentage of labeled mitotic cells should rise to 100% over M phase (Figure 2.2). After S phase, the percentage of labeled mitotic cells fall, which again rise after the total time required for G_1 and G_2 phases. The time

between the midpoint of first ascending and descending curve is the measure of time taken in S phase. On the other hand, the time taken between the midpoint of first and the second ascending curve measure the total cycle time, or the generation time. The duration of autoradiograph varies from cell to cell, but 48 h incubation is sufficient for most of the primary and established cell lines.

In general all cells do not proliferate together. Therefore, the *proliferation index* of growth fraction is calculated by the following equation:

$$N_C/N = [\text{number of proliferating cells/total number of cells}]$$

The time taken to double the number of cells (cell doubling time, T_D) is therefore not equal to the cell cycle or cell generation time. Thus, it is important to carefully distinguish between cell cycle or generation time and the T_D. If all the cells do not have the same generation time, then the T_D is shorter than the generation time. Alternatively, if all cells in the population are not growing, i.e., the growth

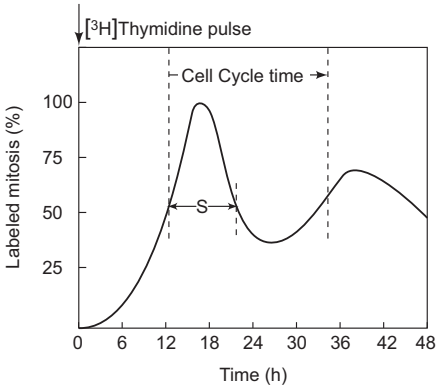

Figure 2.2 Determination of total cell cycle time by autoradiographic technique. [Adapted from Ref. 4]

fraction is less than 1, then T_D will be longer than generation time. The fraction of cells in mitosis, the *mitotic index* (MI) is determined from the autoradiograph by comparing with the total population of cells. Since the location of mitosis is fixed and its duration is short, the proportion of cells in mitosis, the mitotic index can be determined by,

$$MI = \ln 2\ [\text{duration of mitosis/total cycle time}]$$

The most convenient technique to determine the stage of cell division in the cell cycle is carried out by measuring the amount of DNA content in each cell. Progression through S phase and mitosis results in changes of cellular DNA content. The position of cell in the cycle can therefore be determined on the basis of the measurement of DNA content. The cells are treated with a DNA-binding fluorescent dye (e.g., Propidium Iodide, Hoechst 33342, acridine orange) and passed one at a time through a device called *flow cytometer* (see Chapter 9). More the DNA content of a cell, higher is the binding of dye. Thus, by measuring the amount of dye and hence DNA, the stage of each cell in cycle can be determined. Figure 2.3A (left plot) shows pulse width versus area, which distinguishes

between single cells and aggregates. Single cells (G_0/G_1 or G_2/M) will have comparable pulse width values. Whereas, aggregates will have larger width values and can be easily seen on the plot to the right of the single cell region. Single cells have been gated and a typical profile of DNA content of asynchronous mammalian cells is shown in Figure 2.3A (right plot). The cells in G_2 phase have twice as much DNA as the cells in G_1 phase, and the cells in S phase have the intermediate amount. There are clear peaks of G_1 and G_2 cells, but as the cells in S phase are spread over the range of DNA contents, the peak between G_1 and G_2 is quite low. The relative areas of the different peaks, calculated with the help of a dedicated software program, reveal the fractions of the cells in different phases of the cell cycle. The position of cells in cycle explain the physiological status: (a) the unique combination of G_1, S, G_2, and M phase cells signify normal status of cells (Figure 2.3A); (b) presence of quiescent cells is denoted by cells in the G_0 phase (Figure 2.3B); (c) apoptotic cells can be seen by examining the presence of cells in sub-G_0/G_1 phase (Figure 2.3B); (d) heterokaryons can be determined from the position of G_2-M phase cells, for example *tetraploid* cells appear in 4N position, *octaploid* cells appear in 8N position, etc. (Figure 2.3C).

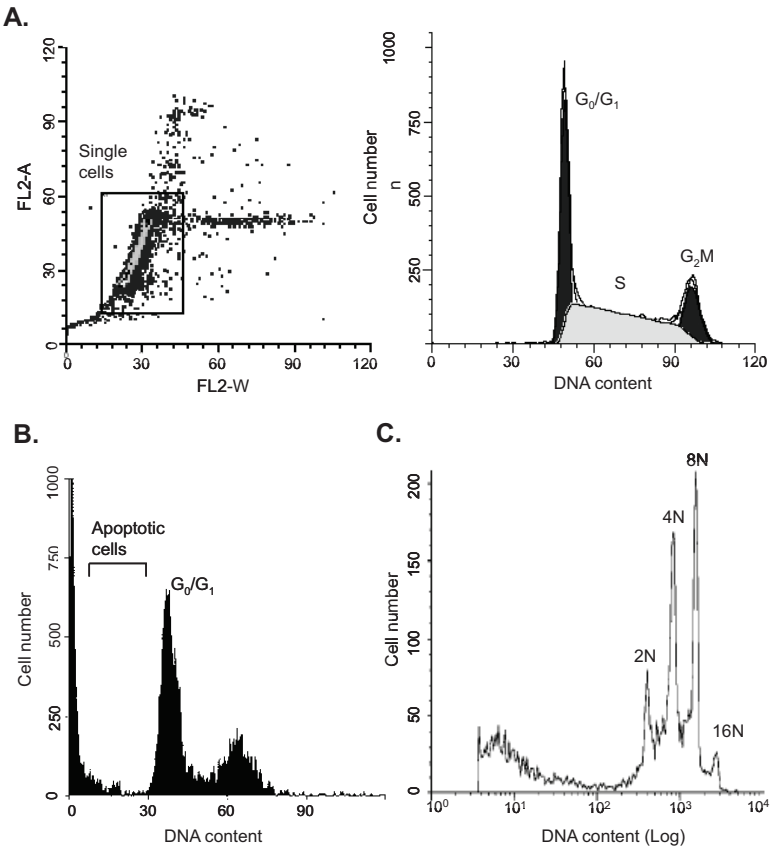

Figure 2.3 Cell cycle analysis of animal cells. **A.** Establishing position of cell cycle histogram; **B.** Resting (G_0) and apoptotic cells (sub-G_0/G_1); **C.** Heterokaryon (2N, 4N, 8N, etc.) in tumor cells.

2.2 GROWTH REGULATION

The duration of cell cycle in somatic cells, is tightly regulated by various factors. In early embryos, the inactivation of maturation promoting factor (mitosis promoting agent) triggers the events that lead to DNA replication and division of microtubule organizing center. In growing somatic cells, the transition in G_1 phase regulates the initiation of DNA replication and division of microtubule organizing center. The growth of mammalian cells is controlled at three different levels [Riley, 1981]:

A. *Intrinsic*: intracellular regulation (cell mass and conformation) to decide whether mitosis will take place or cells will maintain in resting state of G_0 phase.
B. *Intercellular:* space and nutritional factors within a population.
C. *Interpopulation*: hormones, growth factors and inhibitors that act between populations.

Cells divide to control their volume, otherwise their size will become too large. For a long time, it was believed that there is a constant relationship between mass per volume of the cytoplasm and the mass of chromatin that the cells contain. Similarly, in terms of the conformation, it has been observed that in case of normal adhered cells, the growth is related to the degree of flattening on the substratum. For normal *anchorage dependent* cells, there is an optimum height-to-mass ratio for cells to grow in culture. On the other hand, the *anchorage independent* cells (suspension culture) do not change in conformation.

The regulation of growth in mammalian cells can be observed in tissue culture. In establishing primary culture, a fragment of tissue is placed in a plastic dish containing serum-supplemented medium. Within a few days of culture, some cells will migrate away from the tissue and remain attached on the surface of the culture plate for proliferation. The cells growing out of the tissue are called primary cells, and have properties that closely resemble to that of cells *in vivo*. The cells will proliferate only if they are firmly attached to and spread out over the surface of the plate— a property of primary and diploid cell strains termed as anchorage dependence. The cells continue to proliferate until the plate is covered with a single layer (monolayer) of cells (Figure 2.4). Once the dish is fully covered (100% confluent culture), cells stop dividing by a mechanism known as *contact inhibition*. Normal fibroblast (MRC5, WI38) cells are most sensitive to contact inhibition, whereas epithelial and endothelial cells do not stop proliferation even when two growing cells touch each other. As an exception, transformed cell lines (e.g., HepG2, HeLa, MCF-7, etc.) lose contact inhibition and grow in multilayer (Figure 2.4). It is generally believed that when extending cytoplasmic extrusions (*pseudopodia* or *lamellipodia*) of two cells contact each other, they cannot move and the respective cells extend lamellipodia in other directions. For the growth of normal fibroblasts, cellular movement is most important. In confluent culture, since lamellipodia activity and movement are ceased, cells cannot grow. Cessation of growth can also occur due to

which has got no fixed meaning. There is other biological significance for a cell entering into G_0 phase. Often cells enter into G_0 prior to terminal differentiation, they never reenter the cell cycle but instead carry out their function until die. For other cells, G_0 proceeds reentry into the cell cycle. Most of the hematopoietic cells in mammalian systems are found in G_0 phase. However, with proper stimulation, such as encountering with appropriate cytokines or antigen, they can be stimulated to reenter in the cell cycle (G_1) and proceed on to new rounds of mitosis. G_0 phase simply does not represent the absence of signals for mitosis, but also explains the active repression of the genes needed for mitosis. Interestingly, cancer cells do not enter G_0 (ES cells have very short G_0 phase) and are thus destined to repeat the cell cycle indefinitely.

Each normal cell has inherent capacity to rectify DNA damage, if any, during growth process. This observation led to the definition of the *restriction point* or *checkpoint* in the cell cycle. The cell has several stages of checkpoint to interrupt the cell cycle. DNA damage checkpoints occur before the cell enters S phase (a G_1 checkpoint), during S phase, and after DNA replication (a G_2 checkpoint). In spindle checkpoints, the cell detects any failure of spindle fibers to attach *kinetochores* and arrest in the metaphase (M checkpoint), and also detects improper alignment of the spindle itself and blocks cytokinesis. The net outcome of checkpoints is reentry of cells into cycle if the damage is repairable; otherwise triggers apoptosis.

In multicellular organisms, individual cells communicate with each other for coordinated growth, differentiation, and cellular functions. Cell communicates in many ways, most importantly by secretion of soluble growth factors by one cell that interact with the cognate receptor present on the other cell. The binding of growth factors induces many receptor-mediated events (signals) inside the cells, such as activation of *protein kinases*. As a result, many intracellular proteins are phosphorylated and this leads to the transcription of *early response genes* and expression of *transcription factors*. The transcription factors in-turn induce the synthesis of the *delayed response genes*. The most common steps for any cell signaling pathways are described in Figure 2.5A, where activation of cellular response refers to the function mediated by delayed response genes. In response to growth signals, the undifferentiated cells move out of G_0 and enter G_1 phase. The cycle begins in G_1 with increased expression of D1, D2, D3 cyclins, products of delayed response genes. In absence of growth factor or serum, the early and delayed genes are not transcribed, thus the cells do not exit from G_0. In most cells, addition of growth factors/serum induces transcription of early genes, which is maximized within 30 to 60 minutes. However, serum-induced early response genes sharply drop with concomitant increase in the levels of delayed response genes and the corresponding proteins. Thus, turning-on early response genes, followed by delayed response genes and synthesis of corresponding D cyclins are close and interdependent events to activate cell cycle. The profiles for synthesis of early and delayed response genes in mammalian cells, in the absence or the presence of growth factor/serum, are shown in Figure 2.5B.

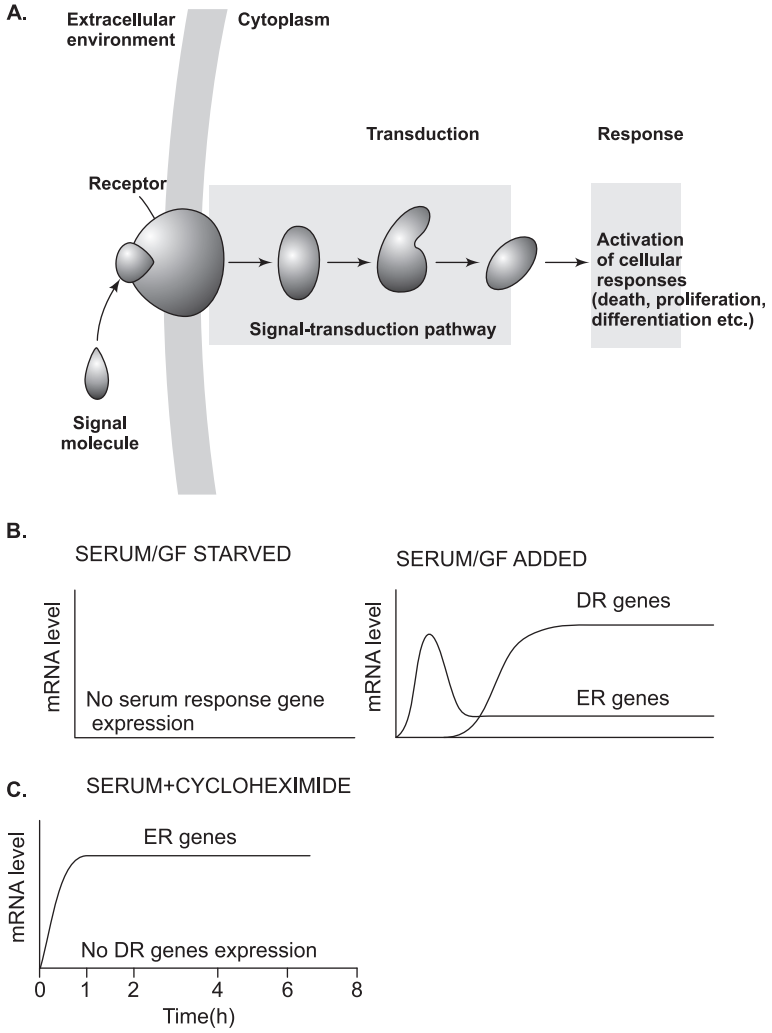

Figure 2.5 Initial events of growth factor/serum mediated proliferation of animal cells. **A.** Major events in cell signaling pathways; **B.** Expression of early response (ER) and delayed response (DR) gene in the presence and the absence of serum. **C.** Drug cycloheximide blocks protein synthesis, thus, DR genes are not turned-on.

To begin cell cycle, D cyclins are associated with cyclin-dependent kinase 4 and 6 (Cdk4 and Cdk6). Cdks are protein kinases that require association with cyclins followed by the phosphorylation for their activity. One of their substrates is believed to be the retinoblastoma gene product (Rb), one of the critical negative regulators of the G_1/S transition. Rb sequesters the E2F transcription factor and inhibits its transactivation (Figure 2.6). Phosphorylation by Cdk4/6 makes Rb dissociate from E2F, allowing it to transcribe number of responder genes that allows the cell to enter S phase. As the cell progresses through late G_1 phase, the

lative population doublings, which generally termed as cellular senescence. In primary cells, senescent may ocurs within few replication cycles. This is also known as *replicative senescence* (RS), the *Hayflick limit*, or the *Hayflick phenomenon* after the name of Dr. Leonard Hayflick, who proposed in 1965.

Hayflick observed that after many doubling this limit is correlated with the length of telomere at the end of a DNA strand. Telomere is a repeating DNA sequence (e.g. TTAGGG) at the 3′ end of DNA, the length of which could be 15,000 base pairs. The function of telomeres is preventing chromosomes from losing base pair sequences at their ends. In the same time, they also stop chromosomes from fussing to each other. At each time of DNA duplication in normal cells, a small strand of DNA 100-200 nucleotides are lost. When the telomere becomes too short, the chromosome reaches a critical length and can no longer replicate. It means that RS is attained and cell dies by a process called apoptosis. The activity to telomere is controlled by two mechanisms: erosion and addition. Erosion takes place each time a cell divides. On the other hand, addition is determined by the activity of telomerase, which elongates chromosomes by adding TTAGGG sequences at the end. In somatic cells, telomerase activity is almost undetectable, whereas in tumor cells and during development fetal cells show its activity. The most common pathways that can trigger senescene in mammalian cells are the p16/Rb and p53/p21 mediated.

2.3.1 Does RS equal for all cell types

RS can be seen in all cell types; however time to enter in this phase is varied among the cell types within a species and from one species to other. The initial work on RS was conducted in fibroblasts, later the phenomenon was seen in other somatic cells, such as lymphocytes, endothelial cells, vascular smooth muscle cells, keratinocytes, etc. Those cells is the body is constantly proliferating may enter senescence much early that the cells that doubles once in a while. *Cumalative population doubling* (CPD) is the number of total doublings is experienced by a cell type before entering into RS. It is believed that CPD has correlation with the longevity of the species, for example somatic cells of long-lived tortoise can divide about 110 times, while hepatocytes of human liver can divide about 50 times and in case of mouse it will be roughly 15 times. The embryonic stem/germ cells and most tumor cell lines (e.g. U938, HeLa, A549, etc.) evade RS, so termed as immortal cells.

2.3.2 Identification of cells undergoing RS

Laboratory grown cultures are heterogeneous, some cells arrest their growth. The proportion of growth-arrested cells is progressively increased until cells stop divide due to RS. Senescent cells can be identified by following properties:

(a) Nonresponsive to growth factors- RS is an irreversible phenomenon, so addition of growth factors cannot stimulate cell division.

Table 2.1 Growth factors, target cells, and signaling pathways for proliferation of animal cells

Growth factor	*Primary activity*	*Major pathways activated*
Platelet-derived growth factor (PDGF)	Promotes proliferation of connective tissue, glial and smooth muscle cells	Ras/MAPK, JAK-STAT
Epidermal growth factor (EGF)	Promotes proliferation of mesenchymal, glial and epithelial cell types	Ras/MAPK, JAK-STAT
Fibroblast growth factor (FGF)	Promotes proliferation of many cell types	Ras/MAPK
Nerve growth factor (NGF)	Promotes outgrowth and survival of neuronal cells	PI3K/Akt
Erythropoietin (Epo)	Promotes proliferation and differentiation of erythrocytes	JAK-STAT
Insulin like growth factor-I (IGF-I)	Promotes proliferation of many cell types	PI3K/Akt, MAPK
Insulin like growth factor-II (IGF-II)	Promotes proliferation of many cell types, primarily of fetal origin	PI3K
Interleukin-3 (IL-3)	Growth of hematopoietic progenitor cells	JAK-STAT
Colony stimulating factors (CSFs)	Growth of hematopoietic progenitor cells	Ras/MAPK, JAK-STAT
Vascular endothelial growth factor (VEGF)	Endothelial cells	PI3K/MAPK

ing. Intercellular signaling is most common in multicellular organisms, and is divided into four types: *endocrine*, *paracrine*, *autocrine*, and *juxtacrine*.

2.4.1 Extracellular signaling and growth factor receptors

Growth factor receptors are named on the basis of ligands, which are usually small polypeptides also termed as growth factors or cytokines. The growth factors have a variety of biological activities. One such activity is stimulation of cell cycle followed by cell division, this will be elaborated here. Some of the commonly used growth factors, their target cells, and corresponding signaling pathways are shown in Table 2.1. The general pathway of cell signaling through ligand-receptor interaction has been shown in Figure 2.5A. Cell signaling process can be divided into three major phases: a) binding of ligand to its cognate receptor, b) cytoplasmic activities where various *adaptor* molecules and kinases bind to the cytoplasmic domain of the receptor and carry out the signaling events, and c) cellular response in the form of gene expression in the nucleus.

The binding of ligand to the cognate receptor monomers causes them to dimerize by the interaction between the extracellular domains. Receptor dimerization takes place by ligand-induced conformational changes in the N-terminal region of type I receptor. The transmembrane domains diffuse laterally bringing the cytoplasmic domains into juxtaposition. Ligand-receptor binding may trigger internal-

ization of the complex by *endocytosis*. In due course of time, the receptors and the ligands are separated; receptor is recycled back to the cell surface for another cycle of receptor-mediated endocytosis. Dimerization of receptors activates the signaling pathway by triggering autophosphorylation of tyrosines at multiple sites present in the cytoplasmic domains of the receptor tyrosine kinase (RTK). The kinase activity of each monomeric receptor phosphorylates the other monomer. Phosphorylation of intracellular domains provides a means for the 'docking proteins' or 'adaptor proteins' to bind to activated receptors. The target of the activated receptors could be a signaling molecule that binds to the adaptor protein, or the target could be an inactive enzyme, which activates upon attachment with the receptor (phosphoinositide-3-kinase), or it could be a substrate (enzyme) that may be activated by phosphorylation (e.g., c-Src). The initial events are depicted in Figure 2.8.

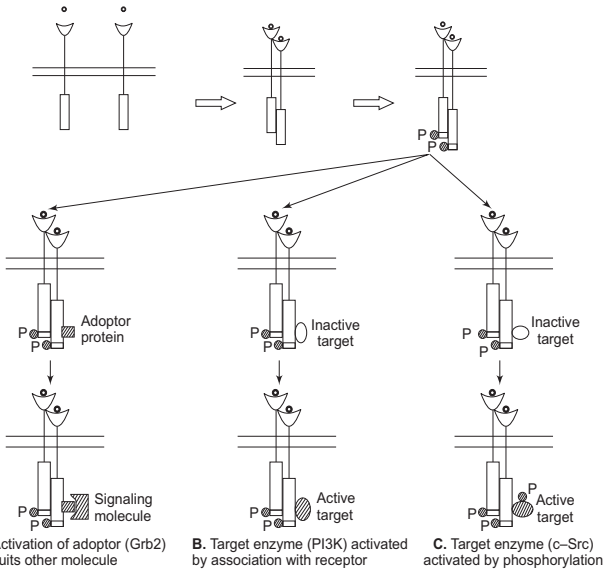

Figure 2.8 Initial events in protein tyrosine kinases induce phosphorylation cascades in animal cells. Cell surface receptors dimerize by interacting with ligand, followed by activation of different cascades. **A.** Activated adaptor protein recruits other signaling molecule(s). **B.** Inactive enzyme gets activated by association with activated receptor. **C.** Activated receptor recruits SH2 domain kinase for its phosphorylation and subsequent activation.

In mammals, EGF and/or PDGF are known to activate different mitogen activated protein kinase (MAPK) pathways through receptor-mediated activation of a cascade of tyrosine phosphorylation and protein-protein association. This activation involves an adapter protein Grb2. SOS is activated by binding with Grb2. Activated SOS in-turn activates small GTP binding protein, Ras. Activation of Ras leads to the activation of the c-Raf (Ser/Thr kinase). The Ras cascade (SOS→Ras→c-Raf) of activation events occur on the cytoplasmic face of the plasma membrane. Raf activates the kinase MEK (formerly known as MAP kinase kinase), which in turn activates MAP kinases (ERK1 & ERK2). The activated MAP kinase (MAPK) enters in the nucleus and phosphorylates specific

2.5 STRESS-INDUCED CELL DEARTH

Protective stress responses to mammalian cells leading to the activation of various pathways that promote survival of cells. For example, *unfolded protein response* increases *chaperon* protein synthesis, which enchances protein folding capacity of the cells thereby promotes cell survival. Beyond certain limit of stress, cells can not cope with the conditions as a result die. The forms of cell death can be explained by programmed (*apoptotic* and *autophagic*) and non-programmed (*necrotic*) mechanisms. Apoptotic and autophagic cell death are induced by activating cellular machinery, whereas necrotic death is induced by injurious agents. Necrosis is an accidental death caused by mechanical damage or exposure to toxic chemicals. Necrotic death is accompanied by distinct characteristic changes including swelling of mitochondria and changes in the ability of the plasma membrane to regulate osmotic pressure. Initially, a progressive hydration of the cytoplasm occurs followed by membrane and organelle disruption, allowing the leakage of the lysosome content into the cytoplasm. Finally nuclear disintegration and complete disruption occur in cell (Figure 2.10). Coagulation necrosis (*oncosis*) may occurs due to denaturation and coagulation of cellular proteins. It can be triggered by factors from outside the cells, such as hypoxia, hyperthermia, physical damage, and toxic injury (Al-Rubeai, 1997). It is characterized by swelling, increase in membrane permeability, and also blebbing.

Apoptosis, by contrast, is a regulated and controlled process operated by preprogrammed manner. This self-demise process occurs in most mammalian cell types and is controlled by a putative 'yin-yang' mechanism involving the activation of killer genes and the deactivation of survival genes (Al-Rubeai, 1997). There are distinct physical changes that occur in apoptotic cells, which include exposure of the phospholipid, phosphatidylserine on membrane surface, shrinkage of cells, bubble-like blebs on their surface, chromatin condensation, release of cytochrome c from leaky mitochondria, and formation of membrane-wrapped fragmented cellular materials (Figure 2.10). Transmission electron micrograph (TEM) of apoptotic cells is shown for better visualization of the changes of organelles in ultrastructure level (Figure 2.11). Apoptosis or programmed cell death is needed for proper development of whole organism or a part of the organism. For example, the formation of the fingers and toes of the fetus requires the removal of the tissue between them by apoptosis. Similarly, the sloughing-off the inner lining of the endometrium layer of uterus at the beginning of menstruation cycle occurs by apoptosis. Programmed death is also needed to destroy cells that represents threat to the integrity of the whole organism. By inducing apoptosis, organism clears cells infected with the viruses: in cell-mediated immune responses the target cells are killed by the process of apoptosis. Apoptosis clears cells containing damaged-DNA, in some in cancer cells are destroyed by apoptosis induced by radiation and chemotherapeutic agents.

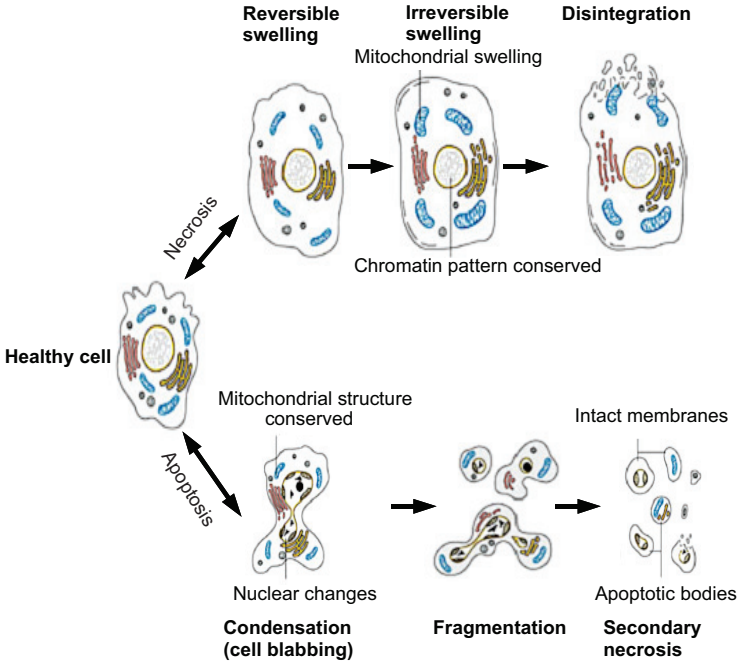

Figure 2.10 Morphological changes in animal cells at different stages of apoptotic and necrotic death [*Source*: Apoptosis, Cell Death, and Cell Proliferation Manual, 3rd edition, Roche Diagnostics, Germany].

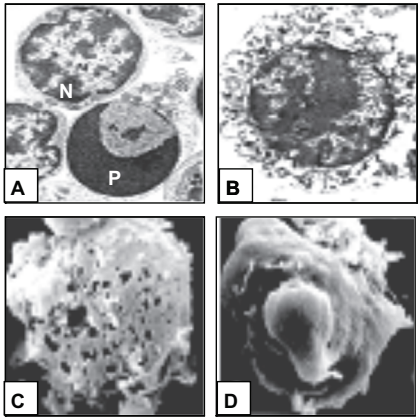

Figure 2.11 Transmission electron micrograph of an apoptotic and a necrotic cell. **A.** Apoptotic (P) and a normal (N) cell. The characteristic chromatin rearrangement appears in P, which is different from its normal organization (N). The membrane and organelles are maintained intact (original magnification: × 8,000). **B.** Necrotic cell, the disruption of plasma membrane and organelles are maintained intact (original magnification: × 10,000). **C.** Numerous lesions appear in the necrotic cell surface (original magnification: × 5,000). **D.** Surface blebbing is evident in apoptotic cell (original magnification: × 5,000). [Falcieri E, et al., (1994) Scanning Microse. 8, 653; Stuppia L, et al. (1996) Cell Death Differ. 3, 397]

state 'grow or die', as they lack G_0 state. High expression of recombinant proteins in cell lines also induces toxic effects to the cells and causes apoptosis. Finally, the accumulation of proteins in side cells due to improper folding leads to apoptotic cell death.

(iv) Virus infection: Like induction of apoptosis in virus-infected cells *in vivo*, mammalian cells undergo apoptosis during production of virus particles in culture.

2.5.2 Mechanisms of apoptosis

Apoptosis is mediated by three possible mechanisms, which are: (i) signals arising within the cells, (ii) signals generated by death activators, and (iii) a mechanism different from (i) and (ii). In all the three mechanisms, mitochondria plays pivotal role in triggering apoptosis. The classical pathway for apoptosis in mammalian cells that are activated by wide variety of stimuli is based on Fas/TNFR1 pathways, as shown in Figure 2.12. Both Fas and TNFR1 have cytoplasmic domains that recruit Fas associated death domain (FADD) and TNF receptor associated death domain (TRADD), respectively, upon activation with the respective ligands. FADD also binds on TRADD. In either case, FADD recruits

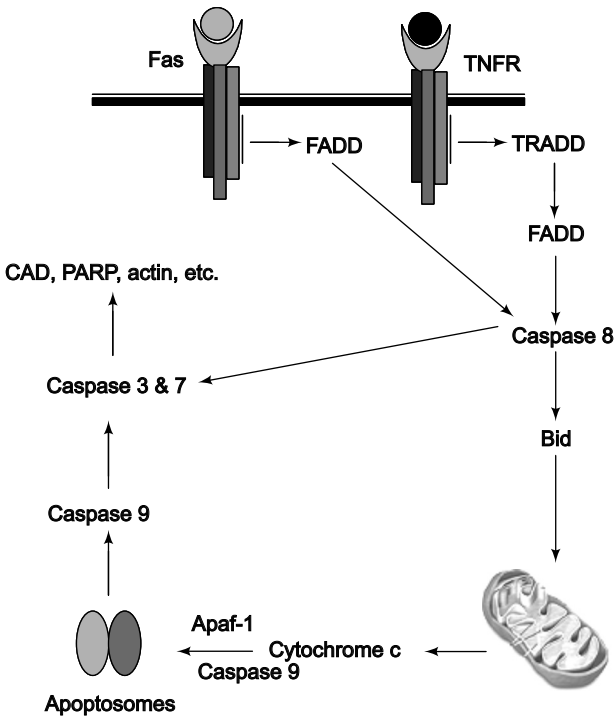

Figure 2.12 Fas/TNFR pathways for apoptotic death of animal cell. CAD: Caspase-activated deoxyribonuclease; Bid: BH3 domain-only death agonist protein; Fas: TNF super family receptor 6; TNFR: TNF receptor.

membrane increases. Annexin-V, a calcium-dependent phospholipid-binding protein, has a high affinity for phosphatidylserine, and binds to the apoptotic cells only (Figure 2.13A).

2. Study of DNA fragmentation by agarose gel electrophoresis: Apoptosis is marked by the change in morphology of the nucleus, together with a distinctive biochemical event like the endonuclease-mediated cleavage of nuclear DNA. Formation of DNA fragments of (180-200 bp) size is a hallmark of apoptosis in many cell types. In apoptotic cells, specific DNA cleavage becomes evident by electrophoresis in agarose gel yielding a typical ladder due to multiple DNA fragments (Figure 2.13B).

3. Analyzing sub-G_0/G_1 fraction of cells: DNA content of apoptotic cells is reduced due to degradation and subsequent leakage from the cells. The lower DNA content means that cells will be stained less with DNA intercalating dye (e.g., PI, Hoechst 33342). Thus, the cells with lower DNA-stained than the normal G_0/G_1 cells (sub-G_0/G_1 peak) are considered apoptotic. The reduction in DNA content of these cells is measured by flow cytometry (Figure 2.13C).

4. In situ DNA fragmentation study by direct terminal transferase DNA labeling reaction (TUNEL): The low molecular weight DNA fragments as well as single strand break (nicks) in high molecular weight DNA can be identified by labeling free 3′-OH termini with modified nucleotides in an enzymatic reaction. Terminal deoxynucleotidyl transferase (TdT), which catalyzes polymerization of nucleotides at the free 3′-OH DNA and ends in a template-independent manner, is used to label cells that have oligonucleosomal nicks/strand breaks in their DNA. The incorporated nucleotides are detected by peroxidase conjugated secondary antibody. After reacting with substrate, the stained cells are identified under a light microscope. Alternatively, fluorescein-labeled nucleotides (FITC-dUTP) can be detected under a fluorescence microscope.

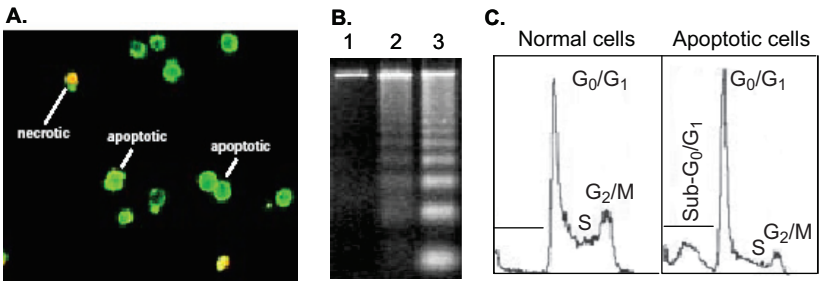

Figure 2.13 Detection of apoptosis in animal cells. **A.** Apoptotic cells appear green due to staining with Annexin-V-FLUOS (green); the necrotic cells take up propidium iodide (PI) and stain orange/green (stained with both Annexin-V-FLUOS and red PI). **B.** DNA fragmentation of apoptotic cells. 1. Control cells, 2. Apoptotic cells, 3. Positive control. **C.** Flow cytometry profiles of DNA content in normal and apoptotic cells stained with propidium iodide. [*Source:* Apoptosis, Cell Death, and Cell Proliferation Manual, 3rd edition, Roche Diagnostics, Germany]

PI	Propidium iodide
PKB/Akt	Protein kinase B
PDGF	Platelet-derived growth factor
PI3K	Phosphatidyl inositol-3 kinase
RTK	Receptor tyrosine kinase
Rb	Retinoblastoma
SOS1	Son of sevenless guanine nucleotide exchange factor-1
SRF	Serum response factor
STAT	Signal transducer and activator of transcript
TEM	Transmission electron microscope
TNFα	Tumor necrosis factor-α
TdT	Terminal deoxynucleotidyl transferase
TRADD	TNF receptor associated death domain
VEGF	Vascular endothelial growth factor

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SUGGESTION FOR FURTHER READING

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