Molecular and Genetic Basis for Tissue Repair and Regeneration

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Jian Xiao, Jiang Wu, Xiaokun Li, Xiaoyan Sun, Biao Cheng, and Xiaobing Fu

3.1 Genes Involved in Tissue Repair and Regeneration

Scientists all over the world are working hard to find genes for tissue repair and regeneration, and hope to achieve the goal of regeneration and repair by regulating several genes. At present, many laboratories in this field have achieved breakthrough results, including Nobel Prize-level results. Chinese scientists, including the team of Xiaobing Fu, have also made important contributions in this field. The important genes involved in tissue repair and regeneration are described below.

3.1.1 The Four Genes of iPS Cells

Academician Xiaobing Fu first reported the reverse differentiation of epidermal cells in the internationally renowned medical journal *Lancet* published on September 29, 2001. In the study of the histological features of healing wounds treated with epidermal growth factors, the academician team found that there were some scattered cell mass positive for β1 integrin and keratin 19 staining in the prickle cell layer and granular layer of the regenerated epidermis. It has an island structure, small cells, few organelles, and no direct histological association with basal stem cells. Therefore, it is preliminarily determined that these β1 integrin and keratin 19 double-stained positive cells in the prickle cell layer and granular cell layer are epidermal stem cells or cells with epidermal stem cell characteristics. On this basis, they conducted a series of identification studies from development, histology, and methodology, and finally confirmed that this stem cell island phenomenon exists only in wounds healed by epidermal growth factor therapy. It is preliminarily proved that the stem cells in the prickle cell layer and granular layer of the regenerated epidermis are reversed by epidermal cell growth factor and is the result of reverse differentiation of differentiated epidermal cells into epidermal stem cells. The publication of this paper has aroused strong repercussions in the academic community. Many researchers in the academic circles thought that the reverse differentiation of mature somatic cells into stem cells was unthinkable until the discovery of induced pluripotent stem cells. It is confirmed that the reverse differentiation of mature somatic cells into stem cells is not only an objective fact, but also a milestone impact on the development of regenerative medicine.

Induced pluripotent stem cells (iPS) are a kind of cells that are reprogrammed to be similar to embryonic stem cells in terms of cell morphology and cell proliferation by introducing pluripotent genes into mature somatic cells or embryonic cells. At present, iPS has been differentiated into various cells such as nerve cells, cardiomyocytes, and cerebral cortical cells. iPS successfully avoided the ethical controversy involved in the study of embryonic stem cells (ESCs) and solved the problem of immune rejection, which is a landmark discovery of stem cell research.

The establishment of iPS cells mainly involves the introduction of several pluripotency-related foreign genes into the differentiated cells, that is, host cells, by virus-mediated or other means. Under suitable culture conditions, these differentiated cells will be transformed into iPS cells. In 2003, Mitsui K et al. found that 20 genes expressed more in embryonic stem cells than in pluripotent cells. Based on this research, in 2006, Shinya Yamanaka of the University of Tokyo in Japan used retrovirus to introduce 24 candidate genes into mouse embryonic fibroblasts and rat tail fibroblasts, and selected four genes Oct4, Sox2, c-Myc, and Klf4 through experiments. These four genes were introduced into

J. Xiao · J. Wu · X. Li
Wenzhou Medical University, Wenzhou, China

X. Sun · X. Fu (✉)
The Chinese People's Liberation Army General Hospital, Beijing, China

B. Cheng
General Hospital of Southern Theater Command of Chinese People's Liberation Army, Guangzhou, China

X. Fu (ed.), *Regenerative Medicine in China*, https://doi.org/10.1007/978-981-16-1182-7_3

mouse embryonic fibroblasts, and induced pluripotent stem cells were successfully obtained. The cells are highly similar to mouse embryonic stem cells in terms of cell morphology and cell proliferation ability.

3.1.1.1 Introduction of iPS Cell Gene Function

Oct4 Gene Function

Octamer-binding transcription factor 4 (Oct4) is a member of the POU transcription factor family and is one of the most important transcription factors involved in regulating embryonic stem cell self-renewal and maintaining pluripotency and cell proliferation. It is also the most important factor in the process of inducing pluripotent stem cell recoding. The human Oct4 gene is located on chromosome 6, and there are 11 subtypes encoding 7 proteins.

Oct4 is a key gene that maintains ESC totipotency and self-renewal and is expressed in unfertilized oocytes. When Oct4 is mutated or knocked out, the embryo cannot form an inner cell mass, the embryoid body undergoes apoptosis, and the embryo loses totipotency in cell. When the Oct4 gene is silenced, mouse and human ESCs differentiate into trophoblast cells. Oct4 forms a network regulatory pathway with Nanog and Sox2 to regulate the totipotency of embryonic stem cells. These three genes maintain embryonic stem cell totipotency and inhibit differentiation through feedforward systems, self-regulatory networks, and other signal transduction pathways.

Sox2 Function

The Sox2 gene is a member of the Sox family, a transcription factor associated with the sex determination gene (SRY) superfamily, and is widely found in the animal kingdom. Sox2 is located on chromosome 3 and is a single exon structure. Sox2 is randomly dispersed throughout the genome and does not form a gene cluster.

The Sox2 gene is one of the important genes for cell reprogramming. It participates in cell formation, maintains embryonic stem cell totipotency and self-renewal, and determines the process of animal gender, nervous system development, and eye development. During embryogenesis, the levels of Oct4 and Sox2 are gradually reduced. As a synergistic gene of Oct4 gene, Sox2 mutation may cause mutation of Oct4 gene and lead to stem cell loss of totipotency. The loss of Sox2 causes the embryo to die shortly after implantation, unable to form trophoblast cells and neuroectoderm.

Klf4 Function

Klf4 is located on human chromosome 4 and has five exons. It is a member of the Klf family and has a multiple tandem zinc finger structure involved in the regulation of cell proliferation and differentiation. Klf4, together with Oct4, Sox2, and c-Myc, regulates stem cell self-renewal and maintains totipotency and participates in the process of human or mouse somatic cells reprogramming.

High expression of Klf4 promotes the increase of Nanog expression and reduces the differentiation ability of cells. Knockout or low expression of the Klf4 gene does not cause embryonic apoptosis, nor affect self-renewal and embryonic development, but can lead to genetic diseases. In ESCs, knocking out Klf4 and Klf2 or Klf4 and Klf5 at the same time results in a decrease in ESC self-renewal ability, but knocking out a gene alone does not change similarly. After the embryo is formed, knocking out Klf2 may cause fatal bleeding and death of embryo in 12–14 days. In the absence of Klf5, the embryo will die due to a lack of trophoblast. Therefore, Klf4, Klf2, and Klf5 are involved in maintaining embryonic development and embryonic stem cell self-renewal. The mechanism of maintaining ESC self-renewal may be related to Toll-Akt pathway, ERK1 and ERK2 enzymes. ERK1 and ERK2 enzymes activate Klf4 gene and phosphorylate Klf4 on Ser123 protein. Phosphorylated Klf4 inhibits Klf4 activity, leading to differentiation of embryonic stem cells.

C-Myc Function

The c-Myc gene is an important member of the cell oncogene, involved in cell proliferation and differentiation regulation, and plays an important role in mouse somatic cells reprogramming and regulates the self-renewal and differentiation of hemopoietic stem cells. The product of c-Myc gene is a 62 kD phosphorylated protein p62c-Myc, a protein consisting of 439 amino acids co-encoded by exons 2 and 3 of the C-one gene, which is located in the nucleus and is a nuclear protein. According to the c-Myc encoded product and functional classification, the c-Myc oncogene is a nuclear protein gene, which has the ability to transform cells and has the characteristics of binding to chromosomes and DNA, and plays a role in regulating cell growth, differentiation, and malignant transformation.

During embryonic stem cell culture, c-Myc expression is reduced when LIF is absent in the culture medium. When c-Myc is highly expressed, ESCs are delayed in differentiation. The mechanism of cell differentiation is related to the LIF-STAT3 pathway. High expression of c-Myc can inhibit the expression of GATA6 gene and reduce the multidirectional differentiation ability of cells. Tomoaki Hishida et al. found that c-Myc, N-Myc, and L-Myc synergistically reduce cell death rate and maintain stem cell totipotency. Maintaining embryonic stem cell totipotency is associated with MAPK and GSK3β pathways, independent of Myc/Max complex. Nakagawa et al. found that knocking out c-Myc during mouse somatic cells reprogramming can increase reprogramming rate and reduce the incidence of tumors.

The Oct4 and Sox2 genes play an important role in the maintenance of pluripotency of embryonic stem cells and are

essential for inducing the reconstruction of iPS cells. These two transcription factors maintain the pluripotency of human iPS cells, while the role of Klf4 and c-Myc is to change the structure of chromatin, thus facilitating the binding of Oct4 and Sox2 and improving the efficiency of induction success. Therefore, Oct4 may be the only independent factor in the reprogramming process, and other factors may only play a synergistic role.

3.1.2 Development-Related Genes

The genes involved in tissue repair and regeneration are closely related to development. Many development-related genes discovered by developmental biologists are used in the research of regenerative medicine. Therefore, we believe that genes related to development are also the focus of regenerative medicine. The academician team believes that developmental biology is the foundation of regenerative medicine. In fact, the repair and regeneration process after tissue damage is also the process of redevelopment of damaged tissues and organs. However, due to changes in seed cells and environmental factors, the repaired and regenerated tissues cannot fully achieve the same structure and function as before the damage.

3.1.2.1 Homeobox Gene

In 1984, the homeobox was discovered, which enabled the important fields of developmental biology and molecular biology to be mechanically combined: regulation of gene transcription can control development. Homeobox gene is a kind of gene closely related to developmental biology. It regulates the growth and development of organisms in time and space. During embryonic development, it gives spatial specificity to the cells in different parts of the body's anterior-posterior axis, thus affecting cell differentiation.

In the following, an important class of homeobox gene Pax family is taken as an example to introduce the close relationship between homeobox gene and tissue repair and regeneration.

The homology of Pax1 and Pax9 is as high as 98%, which plays an important regulatory role in the growth and development of the spine, thymus, parathyroid gland, the third and fourth pharyngeal pouch and their derivatives, and embryonic bone tissues. The effect of these genes on skeletal development are mainly achieved by the following mechanisms: in embryonic development, Bapx1 is widely expressed in the tissue cells of sarcomere and vertebral developmental, Pax1 and Pax9 are essential regulators for the activation of Bapx1 transcription. Knocked out the Pax1 and Pax9 genes during the spinal formation of mouse embryos were completely unable to observe, during development, and the single gene-deficient subjects showed spinal deformity of different degrees.

The Pax2 gene is closely related to the development of kidney. It is one of the earliest genes expressed in the early stage of kidney development. It mainly regulates the proliferation of nephron precursor cells and the formation of mesonephric duct in the early stage. Under ureteric bud induction, Pax2 is continuously expressed in the metanephros interstitial and is active in the metanephros concentrate and epithelial derivatives surrounding the ureter. Pax2 mutant mice can also initially form a ureter, but then the ureter is rapidly decomposed. However, overexpression of Pax2 can accelerate cell proliferation, leading to renal carcinogenesis. Pax8 also has a certain regulatory effect on kidney development.

Pax3 plays an important role in the development of both neural crest cells and melanocytes. Neural crest cells (NCCs) are located on the dorsolateral side of the ectodermal neural tube. They are stem cells with multiple differentiation potential in development. According to their different migration and differentiation directions, they can be roughly divided into nervous system structural cells and melanocytes. Pax3 plays an important role in the development of both types of cells. Pax3 acts as a transcriptional regulator directly regulating the transcription of the melanin-specific gene MITF. The mutation of Pax3 single allele forms Splotch (Sp) mice which white spots on its abdomen due to the loss of melanin. Except for melanin deficiency, the heart, muscle, neural tube, and intestinal nerve center of the homozygous mutant Sp mice are not well developed. Congenital genetic diseases, Waardenburg syndrome (WS) type I and III, are also caused by mutations in the Pax3 gene. Symptoms include shallow hair color, poor hearing, and muscle atrophy of the extremities.

Pax3 and Pax7 are commonly expressed in the early stage of somite development, and then only appear in the skeletal muscle cells of the sarcomere. When the muscle cells differentiate into sarcoplasmic proteins, the expression levels of Pax3 and Pax7 are downregulated; later, Pax3 is strongly expressed in the lateral somites. Both MyoD and MyfS belong to myogenic differentiation factors (MDFs), most of which are active in skeletal muscle precursor cells. RNAi is used to reduce the RNA content of Pax3 and Pax7 in mouse embryos, and the activity of Myf5 and MyoD is inhibited, leading to muscle malformation. Experiments with Sp mice have shown that Pax3 mutation directly affects the formation of muscles in the limbs. Upregulation of the expression of Pax7 can compensate for the loss of Pax3 function within a certain extent, but it cannot play a substitute role.

When Pax7 is knocked out, the mice have normal muscle tissue during embryonic development and birth, and it is seen that Pax7 plays a minor role in muscle formation. In skeletal muscle satellite cells, Pax7, by inhibiting the expression of MyoD and sarcoplasmic protein genes, makes them stay in the stem cell state, so as to redifferentiate in sud-

den events such as muscle damage. Pax3 only plays an auxiliary role. It is speculated that Pax3 is essential for the formation of muscle cells, while Pax7 focuses on the self-repair and regeneration of muscles.

The Pax5 protein, also known as B-cell-specific activator protein (BSAP), is a transcription factor unique to B cells. By binding to B cell development-related genes, it can affect B cell proliferation, homotypic transformation, immunoglobulin gene transcription, and final differentiation.

3.1.2.2 MicroRNA and Development

miRNA is a type of endogenous noncoding protein RNA, a single-stranded small molecule of 20–25 nucleotides in length, widely expressed in multicellular organisms and viruses. After binding to target mRNA, it plays a regulatory role by affecting its translation or degradation. At present, there are more than 2000 miRNAs found in the human body, accounting for 1% of the human genome, regulating 30% of gene expression, finely regulating the expression of target genes of a specific cell and tissue. They are not only involved in the regulation of normal physiological processes in the body, such as cell proliferation, differentiation, development, apoptosis, etc., but also closely related to the occurrence and development of tumors, heart diseases, and neurological diseases. It is predicted that miRNA regulates 30% of human protein-coding genes.

MicroRNA Is Involved in Tissue Development

MicroRNA and lung development studies have demonstrated that it plays a crucial role in many aspects of normal lung development. In 2005, Lu et al. found that the Dicer and AGO protein families are selectively expressed in mouse fetal lung branches, which indirectly indicates that microRNA may be involved in lung development. In 2006, Harris et al. selectively knocked out the Dicer enzyme gene in the endoderm of mice initial lung bud, causing lung dysplasia. MicroRNA was first proposed to play an important role in lung development. In 2010, Dong et al. systematically analyzed the expression profiles of microRNA, mRNA, and protein at different stages of lung development in mice. They found that during lung development, microRNA regulates genes expression mainly by inhibiting translation independent of mRNA degradation.

MicroRNA and Heart Development

With the in-depth study of microRNA, it has been found that microRNAs regulate heart development. MicroRNAs related to cardiac development are mainly microRNA-1 and microRNA-133. Zhao et al. used Cre homologous recombination technology to make mouse heart tissue-specific lack of Dicer, which is necessary for microRNA processing. Mouse heart has many developmental defects, and embryos die early, indicating that microRNA is necessary during heart development. Among them, muscle-specific microRNA expression is affected. miR-1 has two subtypes: miR-1-1 and miR-1-2. miR-1-1 is mainly present in the ventricle. In order to further study the role of miR-1-2 in heart development, the team constructed a mouse model of miR-1-2 knockout and found that miR-1-2$^{-/-}$ mice are prone to large-area ventricular septal defect and die soon after birth. Studies by Lin et al. found that miR-133 plays an important role in regulating cardiac gene expression and function. Experiments show that absence of miR-133a-1 and miR-133a-2 in mouse models causes fatal ventricular septal defect in about half of embryos or suckling mice; surviving mice still develop dilated cardiomyopathy and die of heart failure or sudden death.

MicroRNA and Nervous System Development

miR-124 is one of the most abundantly expressed miRNAs in mature neurons and is widely expressed in neurons of brain, retina, and spinal cord, but is extremely low in undifferentiated neural progenitor cells. Krichevsky et al. found that the phase in which miR-124a began to express was consistent with the phase in which neuronal precursor cells transformed into neural cells and astrocytes and confirmed that miR-124a can change the proportion of cultured embryonic stem cells differentiated into neurons and glial cells. The mature miR-124 sequence is completely conserved from worms to humans. There are three miR-124 genes on different chromosomes in the human and mouse genomes. Overexpression of miR-124 in HeLa cell lines can decrease the expression of more than 100 genes. Krichevsky et al. also found that miR-9 is highly expressed in neurons similar to miR-124a, and experiments have shown that miR-9 can also change the proportion of cultured embryonic stem cells differentiated into neurons and glial cells. Zhao et al. found that the introduction of miR-9 into the embryonic brain by electroporation triggered the early differentiation of neurons. The mechanism by which miR-9 promotes neuronal differentiation is at least partially achieved by inhibiting the nuclear receptor protein TLX. Experiments have confirmed that miR-9 can inhibit the expression of TLX and thus negatively regulate the proliferation of neural stem cells and accelerate the differentiation of neurons.

3.1.2.3 Development-Related Genes and Tissue Repair

Wnt Gene

The epidermis is the outermost layer of the skin and is composed of stratified epithelial tissue. It plays an important role in the defense of animals against external stimuli such as pathogens and water loss. During embryonic development, the role of this barrier has been established, and monolayer epithelial cells differentiate into different stratum corneum. Many human genetic diseases have the characteristic of epi-

dermal destruction, with at least 1 in every 5 patients. Skin regeneration and future treatment require a thorough understanding of the molecular mechanisms of the epithelial stratification process. Wnt ligand protein plays an important role in the process of inducing skin hair follicles and in the self-renewal of epidermal stem cells between adult hair follicles. However, during embryonic development, we know little about the role of Wnt signaling in epithelial stratification. In the research of Professor Zhang Zunyi's team, by using the mouse genetic model to block the secretion of Wnt signal in the epidermis during embryonic development, it was found that the Wnt signal generated by the epidermis can activate the dermal BMP-FGF signal axis, and the latter re-feedback regulated the stratification of the epidermis. The findings of this study identified a genetic hierarchy of signaling that plays a necessary role in epidermal-dermal interactions and promotes understanding of mammalian skin development.

Lin28a Gene

Professor George of Harvard Medical School reported on *Cell* that by reactivating a latent gene called Lin28a, hair, cartilage, skin, and other soft tissues of model mice can be regenerated. Lin28a can partially enhance tissue repair by enhancing mitochondrial metabolism, which provides new insights into the development of novel regenerative therapies. Lin28a was first discovered in worms. It is abundant in embryonic stem cells and is particularly strongly expressed during early embryogenesis. It has been used to reprogram skin cells into stem cells, and can also bind to RNA to regulate gene expression. In order to better understand the role of Lin28a in promoting tissue repair, the researchers observed which specific RNAs can bind to Lin28a and subsequently discovered a small RNA, Let7, which promotes cell maturation and aging. Lin28a enhances the production of metabolic enzymes in mitochondria and stimulates tissue to regenerate by altering the metabolism of mitochondria to produce more energy. Since Lin28a is difficult to introduce into cells, tissue repair can be achieved by directly activating mitochondrial metabolism. Direct activation of mitochondrial metabolism by using a small molecule compound can also enhance the healing rate of the wound, which suggests that tissue regeneration can be induced by some tissue repair drugs. However, Lin28a does not induce regeneration of all tissues, and Lin28a may be a key factor in the composition of healing compounds.

Smed-Prep Gene

The worm has an extraordinary ability to regenerate when it is cut off, and these parts even include the head and brain. Professor Aziz Aboobaker of the University of Nottingham in the UK reported on *PLoS Genetics* that a homeobox gene called Smed-prep plays an important role in the regeneration of worms. This study may make it possible to regenerate aging or damaged human organs and tissues. Smed-prep is an essential factor for the proper differentiation and localization of the cells that make up the worm's head and is the key to determining the position of the head. Although the emergence of Smed-prep is the determining factor to keep the head and brain in the correct position, worm stem cells will form brain cells under the influence of other irrelevant genes. Without Smed-prep, the relevant cells cannot organize themselves to form a normal brain.

3.1.3 Tumor-Related Genes

In the paper *Developmental biology and comparative biology: the important research fields in wound repair and regeneration*, Fu Academician pointed out: from the perspective of biological processes, wound repair and tumor formation are both the results of cell proliferation, differentiation, and angiogenesis; only the former is controllable and the latter is out of control. In the past, it was suggested that the formation of tumor is a result of overexpression and action of proto-oncogenes such as c-fos and c-jun caused by some gene mutations. However, many studies have suggested that proto-oncogenes or their proteins are also highly expressed or their activities are upregulated in different stages of embryonic development and in some important organs and tissues, and these tissues have no tumor formation. Conversely, during the rapid proliferation of such cells, the healing of the wound is scar-free. Therefore, the high expression of these proto-oncogenes in embryonic tissues without scars, cancer, and tumorigenesis also suggests that there may be similar and different regulatory mechanisms in normal cell proliferation and differentiation, uncontrolled cell proliferation and differentiation (such as uncontrolled growth of cancer cells), embryonic development, and postnatal repair. An understanding of these regulatory mechanisms may help us find a switch that regulates wound healing. Therefore, tumor-related genes, especially proto-oncogenes and tumor suppressor genes, are also closely related to tissue repair and regeneration.

3.1.3.1 Proto-Oncogene

Proto-oncogene is a gene that controls cell growth in cells. It is a normal gene related to cell proliferation under normal conditions. It can stimulate cell growth to meet the requirements of cell renewal. It is highly conserved in evolution, and its expression products are extremely important for the physiological function of cells. More than 100 proto-oncogenes have been identified. When the structure or regulatory region of the proto-oncogene is mutated and the gene expression product is increased or the activity is enhanced, the cell will continue to grow or protect the cell from death without receiving the growth signal and finally cause cell carcinogenesis.

The proteins encoded by proto-oncogenes are known to be involved in many factors regulating cell growth, and these factors are involved in the regulation of cell growth, proliferation, and differentiation pathways. Proto-oncogene expression products are classified into the following four categories according to their role in the cellular signaling system.

Extracellular Growth Factor

Extracellular signals include growth factors, hormones, neurotransmitters, drugs, etc., which act on the receptor system on the cell membrane or are directly delivered to the cell, and then activated by various protein kinases to phosphorylate the transcription factor, triggering transcriptional activation of a range of genes. It is through this pathway that the Sis gene works. It is known that P28 protein encoded by v-Sis gene and human c-Sis gene is homologous to the β-chain of platelet-derived growth factor (PDGF). When the Sis gene expression forms a dimer like PDGF, it acts on the PDGF receptor and makes phosphatidylinositol in the cell membrane catalyzed by the corresponding kinase to form phosphatidylinositol-4, 5-bisphosphate (PIP2). Under the action of phospholipase C, PIP2 is hydrolyzed to generate diglyceride (DG) and inositol triphosphate (IP3), activate protein kinase C, transform the recipient cells, and stimulate intracellular receptor synthesis. It is indicated that the Sis gene is related to PDGF and its function is very similar. In addition, C-Sis expression protein P28 promotes vascular growth in the same way as PDGF.

Transmembrane Growth Factor Receptor

Another type of proto-oncogene product is a transmembrane receptor that accepts extracellular growth signals and transmits them into the cell. The transmembrane growth factor receptor has a cytoplasmic structural region and has tyrosine-specific protein kinase activity. Many proto-oncogene products also have this enzyme activity, such as c-src, c-abl, and the like. The kinases encoded by other proto-oncogenes (c-mos and raf) phosphorylate serine and threonine residues rather than tyrosine. By this phosphorylation, its structure is changed, the activity of the kinase on the substrate is increased, and the intracellular transmission of the growth signal is accelerated.

Intracellular Signalosome

After the growth signal reaches the intracellular, a series of intracellular information transmission systems are used to transmit the received growth signal from the intracellular to the nucleus to promote cell growth. Most of these transmit system members are products of proto-oncogenes, or affect the second messenger (cAMP, diglyceride, Ca^{2+}, etc.) by the action of these gene products. Oncogene products as intracellular signalosome include non-receptor tyrosine kinases (c-src, c-abl, etc.), serine-threonine kinases (c-ras, c-mas), ras proteins (H-Ras, K-ras and N-ras, etc.), and phospholipase (crk product).

Nuclear Transcription Factor

It is known that certain proto-oncogene expression proteins (such as myc/fos, etc.) are localized in the nucleus, and they bind to regulatory elements of the target gene to directly regulate transcriptional activity as a transcription factor. These proteins are usually expressed rapidly when cells are stimulated by growth factors, promoting cell growth and division. It is now widely accepted that c-fos is an immediate-early gene (IEG).Under the action of growth factors, phorbol esters and neurotransmitters, c-fos acts as the third messenger to transmit information and can be expressed instantly and transiently.

3.1.3.2 Tumor Suppressor Gene

A tumor suppressor gene or an anticancer gene is a type of gene that can inhibit the activity of proto-oncogene. When it is activated, it inhibits cell proliferation and plays an important role in regulating cell development, growth, and differentiation. When various reasons such as mutation cause gene inactivation or the product is inactivated, the inhibition disappears, which may lead to tumor formation and canceration.

Since the study on isolation and identification of tumor suppressor genes is later than proto-oncogenes, only the mechanism of Rb and p53 is well understood.

Retinoblastoma Gene (Rb Gene)

The Rb gene was the first tumor suppressor gene discovered in children's retinoblastoma; hence, it is called Rb gene. Under normal circumstances, retinal cells contain active Rb gene, which controls the growth and development of retinal cells and the differentiation of visual cells. When the Rb gene loses function or congenital deletion, retinal cells proliferate abnormally and form retinoblastoma. Inactivation of Rb gene is also common in many tumors such as osteosarcoma, small cell lung cancer, and breast cancer, indicating that the anticancer effect of Rb gene has a certain extent.

The Rb gene is relatively large, located in human chromosome 13q14, contains 27 exons, transcribes 4.7 kb mRNA, and encodes protein P105. The protein is located in the nucleus and has two forms of phosphorylation and non-phosphorylation. The non-phosphorylation form is called active type and can promote cell differentiation and inhibit cell proliferation. Experiments have shown that after the introduction of Rb gene into cells of retinoblastoma or osteosarcoma, the growth of these malignant cells was inhibited. Significantly, the phosphorylation of Rb protein is closely

related to the cell cycle. For example, lymphocytes in quiescent state only express non-phosphorylated Rb protein. Under the induction of mitogen, lymphocytes enter S phase, and Rb protein phosphorylation level is increased. Terminally differentiated monocytes and granulocytes only express high level of non-phosphorylated Rb protein. Even under the induction of growth factors, Rb protein did not phosphorylate, and cells did not divide. It is suggested that cell growth stops, Rb protein is at a low phosphorylation level, and tumor cells in proliferation only contain phosphorylated Rb protein. It indicates that the phosphorylation modification of Rb protein plays an important role in regulating cell growth and differentiation.

P53 Gene

The human p53 gene is located at 17P13, has a full length of 16 ~ 20 kb, contains 11 exons, and transcribes 2.8 kb mRNA. The encoded protein is p53, which is a nuclear phosphorylation protein. The p53 gene is the most associated with human tumors. In the past, it has been regarded as an oncogene. It was not until 1989 that mutant p53 was known to play the role of oncogene. Later, it was confirmed that wild-type p53 is a tumor suppressor gene. The p53 gene expression product p53 protein consists of 393 amino acid residues and exists as a tetramer in vivo with a half-life of 20–30 min.

Under normal conditions, the p53 protein content in cells is very low, and because its half-life is short, it is difficult to detect. However, in the growing and proliferating cells, it can be increased by 5–100 times. Wild-type p53 protein plays an important role in maintaining normal cell growth and inhibiting malignant proliferation, and is titled "Geneguard." The p53 gene constantly monitors the integrity of the gene. Once the DNA of the cell is damaged, the p53 protein binds to the corresponding part, acts as a special transcription factor, activates the transcription of the p21 gene, arrests the cell in the G1 phase, inhibits the activity of the unwinding enzyme, interacts with replication factor A, and participates in DNA replication and repair. If repair fails, p53 protein initiates a programmed death process to induce cell suicide and prevent the formation of cancer-prone mutant cells, thereby preventing cell malignant transformation.

3.2 Protein Molecules Involved in Tissue Repair and Regeneration Regulation

3.2.1 Growth Factor Protein

As early as 1991, Academician Xiaobing Fu and his team began the leading research on modern wound repair, edited and published the first book on *growth factors and wound repair* in the world, Growth Factor and Wound Repair. Since the 1990s, Academician Xiaobing Fu led the team to systematically carry out research on growth factor-regulating wound healing in China and first reported the epidemiological characteristics of chronic refractory wounds on human body in China.

Academician Xiaobing Fu pointed out: "Studying the molecular biological mechanism of wound healing and applying growth factors to wound repair and treatment is a major advance in trauma medicine since the 1980s. It not only leads to a profound change in the concept of wound treatment, but also brings revolutionary breakthrough in wound healing." Professor Li Xiaokun, a leading scientist in the development of new growth factor drugs in China and a distinguished professor of Changjiang Scholars, has been working closely with the Academician Fu's group since the 1990s to lead the largest research team of fibroblast growth factor in China. They found out the way to engineering growth factor gene drugs, which made China the first country in the world to develop fibroblast growth factor into clinical drugs, and made outstanding contributions to accelerating the development of new biological drugs with China's independent intellectual property rights.

3.2.1.1 FGF—Fibroblast Growth Factor

As one of the most important regulatory factors in the repair of tissues such as skin, hair follicles, and sweat glands, it is of great clinical and social significance to develop fibroblast growth factor as an innovative drug that shortens the healing time of wounds and improves the quality of wound healing.

In 1996, Professor Li′s team solved a series of engineering and technical problems that have long restricted the industrialization of FGF. They are the first to develop FGF series of innovative drugs in the world, which are widely used in the treatment of burn, trauma, and diabetic complications. The team's main contributions to FGF drug development are as follows.

Establish FGF-Efficient Secretion Expression System

Before the 1990s, genetic engineering drugs have long faced a series of engineering and technical problems such as low protein expression, misfolding, easy formation of inclusion bodies, difficulty in large-scale preparation and purification, poor stability, and short half-life, which severely restricted the development and transformation of protein drugs. The team screened and obtained the SecB gene of *E. coli* secretion factor, which was used as a molecular chaperone for FGF secretion expression vector, constructed the recombinant expression plasmid pT7-SecB-FGF, established a high-efficiency secretion expression system of FGF, and realized the soluble expression of protein [1]. The expression reached more than five times the international level in the same period.

The First Structural Transformation of FGF Successfully Solved the Technical Problems of Large-Scale Production Process

FGF2 easily forms intermolecular disulfide bonds, and it is easy to form dimers or polymers during expression to cause precipitation. Through structural analysis, Cys78 and Cys96, which are not in the active region, were mutated to Ser, and the highly expressed human mutant rhFGF2 was successfully constructed. Its expression level and stability were significantly higher than wild-type rhFGF2. The bioinformatics technique was used to analyze the mRNA of full-length FGF1, and it was found that it was easy to form a hairpin loop structure in the ribosome binding site, which affected the initiation of translation and resulted in a low expression level of full-length FGF1. Based on the interaction characteristics between protein and receptor, the 19 amino acids at N-terminal were knocked out on the basis of the retained protein activity, and the genetically engineered strain with high soluble expression of rhFGF1135 was successfully constructed, and the expression amount reached 108 mg/L, which satisfied the industrial production demand.

Created FGF Large-Scale Preparation Process and Quality Standards

The growth and expression patterns of *E. coli* engineered bacteria loaded with FGF plasmid were found, and the production technology of fed batch high-density fermentation was established [2]. The high-efficiency two-step separation and purification process was established based on the characteristics of the specific binding of FGF and heparin. The purity of the target protein reaches 99%, and the protein recovery rate is increased by 50%. For the defects of poor stability and short half-life of the recombinant protein in vitro and in vivo, a specific affinity fixed-point solid phase modification technology was developed to form a standardized technical system for industrial production of FGF, and a standard activity assay for the determination of FGF-promoting cell proliferation by NIH3T3 cell line/MTT method was established. The FGF production and quality control technical standards were included in the third part of the 2005 edition of *Chinese Pharmacopoeia*, which is the first production and verification regulation for growth factor genetic engineering drugs.

Developed the First Drug-Loaded Class III Implantable Medical Device that Combines FGF with Tissue Engineering Materials

A variety of drug-loading materials were screened, and the rhFGF2-collagen composite active material suitable for fistula, bedsore, uterine erosion, and the like was successfully developed for the first time. The production process and activity standard of the biomaterials were first established, and the sponge-like freeze-drying molding technology was optimized. The material was selected into the catalogue of foreign international assistance of the Ministry of Commerce of the People's Republic of China and the catalogue of war storage of the entire military. It is used for national defense and military first-aid equipment, and became an important strategic military product with independent intellectual property rights in China.

At the same time, Professor Li Xiaokun's team has carried out a lot of pioneering work around the development and mechanism of new endocrine FGF subfamily innovative drugs. It is the first report that the upregulation of FGF21 in diabetic patients through clinical practice, further reveal that FGF21 plays a role in the metabolism of glucose and lipid by activating FGFR and PPAR-adiponectin, and systematically elucidate the role of FGF21/FGFR in fatty liver and atherosclerosis. It has internationally firstly analyzed the complex structure of FGFR phosphorylation substrate PLCγ. Based on the above research, a series of high-level academic papers have been published in the international authoritative magazines such as *Cell Metab*, *Circulation*, *Mol Cell*, *JACC*, *J Hepatol*, *Biomaterials*, *Diabetes*, etc. as corresponding authors, in which the research on the role of FGF in metabolism was selected as one of the top ten breakthroughs of *Cell Metab* in the past 10 years. The above basic research work lays a theoretical foundation for screening, designing, and developing new growth factor drugs.

FGF series proteins can significantly accelerate the healing rate of burn wounds, bedsore, diabetic ulcers, flap transplantation, fistula, and other wounds, can significantly inhibit scar formation, reduce epidermal water loss, reduce skin cuticle thickness, and promote the wound skin accessory organs such as sweat glands, hair follicles, and nerve regeneration and repair. The long-term follow-up observation on the clinical application of FGF new drugs showed no adverse reactions such as hyperplasia and abnormal hyperplasia. Clinical application showed that FGF new drugs have changed the traditional treatment methods based on anti-infection in the process of wound repair and provided a safe and effective new treatment of active repair and functional repair for wound repair and tissue regeneration.

Through the above-mentioned series of technological innovations, Professor Li′s team has taken the lead in solving the series of genetic engineering technical problems in the process of FGF family protein pharmaceutical production in the world. The team has developed a new class of bio-products with independent intellectual property rights, namely, bovine bFGF fusion protein, human bFGF, aFGF modifier, together with a class III implantable medical device containing FGF, which provides new therapeutic drugs for severe trauma, ulcer, and diabetic complications with a cumulative output value of over 4 billion yuan. And the team has established the first National Engineering Research

Center for genetic engineering drugs, which promoted the improvement of engineering technology in related fields in China.

3.2.1.2 PDGF—Platelet-Derived Growth Factor

Platelet-derived growth factor (PDGF) is a basic protein normally stored in platelet alpha granules, which is secreted by various cells, such as macrophages chemotactic to the damaged site, smooth muscle cells at the damaged vessel, and vascular endothelial cells in the damaged area. PDGF has many important roles in embryonic development, cell differentiation, and response to tissue damage. It is one of the early growth factors in wound healing. In particular, it has significant healing effects on some chronic refractory wounds, such as diabetic ulcers, chronic venous ulcers, bedsores, and radiation ulcers.

Becaplermin (recombinant PDGF-BB) is currently the only growth factor drug approved by the FDA for the treatment of diabetic foot ulcers. In addition, rhPDGF-BB developed by a company has entered clinical phase III trials for pressure ulcers and venous ulcers. After more than 10 years of market application, rhPDGF-BB has been clinically proven to be safe and effective. rhPDGF-BB has a significant effect on the repair of pathological deep ulcer wounds and the promotion of skin vascular regeneration [3].

3.2.1.3 NGF—Nerve Growth Factor

In the 1960s, Levi-Montalcini and Cohen isolated and purified a soluble protein that promotes nerve growth and named it nerve growth factor (NGF). The discovery of NGF has led to the recognition that in the development of the nervous system, some factors that promote the development and growth and maintain the activity of neurons are needed, thus opening up a new field of neurobiology. These two scholars won the Nobel Prize in Physiology or Medicine in 1986. The current clinical use of NGF is taken from the submandibular gland of mice. Numerous basic and clinical trials have confirmed that NGF plays an important role in the treatment of TBI and stroke.

NGF exerts neuroprotective effects by blocking the cascade of secondary damage to nerve cells. Its neuroprotective mechanism is as follows:

(a) Antagonizing the toxicity of excitatory amino acids: the increase of excitatory amino acids such as glutamate can lead to calcium overload, activate the apoptotic signaling pathway to allow neurons to enter the apoptosis program. NGF can inhibit the increase of glutamate, block calcium overload, and prevent neuronal apoptosis.
(b) Reducing oxygen free radicals: NGF can increase the activity of oxygen free radical scavengers such as catalase and superoxide dismutase, and reduce the peroxidation damage of neurons.
(c) Reducing the cytotoxicity of nitric oxide (NO): NO can inhibit the activity of oxidative phosphorylation enzyme, thereby inhibiting the respiratory function of cells. NGF reduces the cytotoxicity of NO by reducing the activity of NO synthase.
(d) Stabilizing intracellular Ca2+ concentration: NGF can regulate protein expression and function related to calcium influx, stabilize Ca2+ concentration, and inhibit neuronal damage caused by calcium overload.
(e) Inhibition of apoptosis protein activity: NGF can inhibit the activity of apoptosis proteins, inhibit the activity of pro-apoptotic proteins and apoptosis-executing proteins, and then inhibit neuronal apoptosis.

3.2.2 Important Proteins Involved in the Regulation of Tissue Regeneration Found in Lower Organisms

The tissues of various organisms on the earth have different regenerative abilities. This varying degree of tissue regeneration is formed after the long-term evolution of the organism. In general, the regeneration ability of lower biological tissues is stronger than that of higher organisms. Tissues with lower differentiation have stronger regeneration ability than tissues with higher differentiation. Tissues that are usually vulnerable to damage and tissues that are frequently updated under physiological conditions have strong regeneration ability. On the contrary, the regeneration ability is weak or lacking. In general, lower organisms (including plants) have a strong ability to regenerate. Higher organisms, such as humans, have weaker ability to regenerate, and generally only heal wounds and cannot reproduce a certain limb or an organ. Humans also have a certain ability to regenerate, but extremely limited. Some of the lower organisms have a strong ability to regenerate: the tail of the gecko, the limbs of the salamander, the foot of the crab can be reformed after being lost, and the sea cucumber can form all the internal organs. In contrast, human regeneration capacity is very limited.

3.2.2.1 Pax6 Gene Regulatory Proteins and Lens Proteins Found in the Lens

The expression order of related proteins in the lens regeneration of the *Bufo raddei* tadpole is similar to that of the salamander. The expression order of proteins is β-crystallin, γ-crystallin, and α-crystallin, and the latter two proteins are expressed almost simultaneously. In the early stage of *Bufo raddei* tadpole regeneration, the pigment cells in the dorsal and ventral edge of the iris undergo dedifferentiation and cell proliferation, and Pax6 gene is expressed in the dorsal and ventral edge of the iris. During the differentiation of the

regenerated lens cells, the Pax6 gene is expressed in the lens epithelial cells. In the process of lens regeneration, Pax6 gene is expressed in the retina, ciliary body region, cornea, and lens epithelium, and is mainly expressed in the ganglion cell layer, the inner nuclear layer, and the outer nuclear layer of retina. The expression of Pax6 gene is closely related to the regulation of lens regeneration. βB1 lens protein is an important structural protein distributed in the lens of animals. Both Pax6 and Prox1 can regulate the expression of βB1 lens protein. The expression of βB1 lens protein was detected by immunofluorescence technique after 21 days of lens regeneration in *Bufo raddei* tadpole. The experimental results further confirmed that the lens regeneration of *Bufo raddei* tadpole experienced dedifferentiation, cell proliferation, and cell redifferentiation of pigment epithelial cells on the dorsal edge of iris. In the process, both the regenerated lens and the normal lens expressed the βB1 lens protein. This research suggests that lens-associated proteins play a crucial role in the lens regeneration of lower organisms.

3.2.2.2 Genes and Proteins Related to Nerve and Spinal Cord Regeneration

The zebrafish's nervous tissue has a strong ability to regenerate. Studies have shown that throughout the life of the zebrafish, its retina and other nerve tissues have been continuously undergoing metabolism of new and old cells, which means precursor cells with neurogenesis ability are always present in zebrafish. Regeneration of the retina and optic nerve was first used to study the regeneration of the zebrafish nervous system. During the regeneration of the retina, Muller glia cells play a huge role. And for the molecular signaling pathways that activate the cells, researchers have discovered numerous genes such as hspd2, msp1, mdka, mdkb, stat3, and signaling pathways such as Wnt, Notch, and FGF all play an important role in it. In recent years, studies on the regeneration of zebrafish spinal cord have been gradually carried out. In the regeneration of spinal cord, the difference in the regenerative capacity of different brain nuclei is obvious, and this difference depends on the location of spinal cord injury, suggesting the molecular mechanism controlling its regeneration is complex. Studies have found that cell adhesion proteins L1.1, GAP-43, cAMP, and other factors play a major role in spinal cord regeneration.

3.2.2.3 HP1-1-Related Proteins and Mcm5 Pathway Regulate the Related Proteins of the Planarian Regeneration

Freshwater planarian also has a strong ability to regenerate, and when the body is cut to 1/279, it can still regenerate an intact individual. The regeneration potential of the planarian is mediated by its abundant adult stem cells, which initiate proliferation, migration, and directional differentiation to participate in the regeneration after the damage of the planarian. Scientists have discovered the role and function of 205 epigenetic factors in the regeneration of planarian, 12 new stem cell regulatory factors, and revealed that the epigenetic regulation mechanism of adult stem cells of the planarian is highly similar to that of higher organisms. They found that the heterochromatin protein HP1-1 is specifically expressed in the planarian stem cells and maintains self-renewal of stem cells. After the injury, HP1-1 promoted the regeneration and proliferation of stem cells. Unlike the previous reports of major involvement in heterochromatin formation and gene silencing, the molecular mechanism of Hp1-1 involved in the regeneration is mainly through promoting transcription elongation, and Mcm5 is an important downstream target gene, which is highly enriched and expressed in the stem cell group of planarians. HP1-1 can bind to the proximal promoter region of Mcm5 like the activated RNA polymerase after injury, thereby activating Mcm5, leading to upregulation of Mcm5, which is beneficial to the regeneration of planarians.

3.2.3 Tumor-Associated Tissue Regeneration Regulatory Proteins

With the research on tissue regeneration regulatory proteins, some tumor-regulated tissue regeneration regulatory proteins, including paxillin, focal adhesion kinase (FAK), midkine (MK), mammalian target of rapamycin (mTOR), have been discovered. They also play an important role in the regulation of tissue repair.

3.2.3.1 Paxillin

Paxillin is a cytoskeletal protein involved in actin-membrane attachment at the site of adhesion (adhesion) of extracellular matrix cells. Paxillin plays an important role in the signaling of integrin, and the reorganization of cytoskeleton mediated by integrin requires phosphorylation of the paxillin tyrosine residues. Paxillin is phosphorylated by focal adhesion kinase (FAK) at its 118th tyrosine residue. Due to its special structure and function, paxillin has gradually attracted the attention of researchers. Many studies have shown that paxillin as a junction in cells can bind to a series of structural proteins and signal proteins and participate in cell migration activities associated with damage repair, embryo development, and tumor metastasis. Paxillin not only participates in the assembly of focal adhesions, but also plays an important role in cell morphological change, movement, adhesion, and cell signal transduction.

3.2.3.2 Midkine

The midkine (MK), a heparin-binding growth factor, is a low molecular weight protein. MK is widely distributed in tissues during embryonic period, but it is limited to certain specific sites in adults. There are many kinds of MK receptors

and the complex signaling pathways, which determine the diversity of MK functions. It can promote the growth, survival, differentiation, and migration of many cell types, and has antiapoptotic effects. It is not only closely related to tumorigenesis, but also involved in the development of many tissues and the repair and regeneration process after injury. A large number of MK expression can be detected in the early stages of tissue damage and repair processes such as cerebral infarction, peripheral nerve injury, spinal cord injury, fracture, myocardial infarction, and skin damage.

MK is not expressed in normal adult bone tissue. When the mouse's tibia fractures, MK expression can be detected during bone repair. After transfection of MK cDNA into ATDC5 chondrocytes, MK was overexpressed in the cells. As a result, most of the transfected cells showed stronger cartilage-forming ability, and they synthesized more sulfated mucopolysaccharides, aggrecan, and type II collagen. These results indicate that MK not only participates in bone formation and repair after injury, but also plays an important role in cartilage formation.

3.2.3.3 Mammalian Target of Rapamycin

Mammalian target of rapamycin (mTOR) belongs to the phosphatidylinositol kinase-associated kinase (PIKK) superfamily and is a class of evolutionarily highly conserved protein kinases widely present in various biological cells. mTOR is an effect protein downstream of the phosphatidylinositol-3 kinase (PI3K)-Akt signaling pathway. It plays a key role in cell survival and participates in various biological processes such as gene transcription, protein translation initiation, and apoptosis under the activation of various factors. The main functions of the PI3K-Akt-mTOR signaling pathway can be summarized as the regulation of the synthesis of various proteins, cell growth, proliferation, and apoptosis. The signal from the mTOR protein is essential for the unique tissue regeneration of the planarian, which is common in humans and most mammals. The flatworm has the ability to regenerate cells, and the inactivation of mTOR protein prevents the regeneration of flatworms. This also indicates that inactivation of mTOR protein in mutant cells can also inhibit the growth of cancer cells.

3.3 Stem Cell Niche

In addition to self-renewal, adult stem cells produce daughter cells that maintain homeostasis by constantly producing and replacing short-lived and highly differentiated cells in blood, skin, midgut, nerves. Whether the stem cells are self-renewing or differentiation, this decision process is carefully controlled. If too many progeny cells begin to differentiate, the stem cells will decrease. In another case, unregulated self-renewal will increase the number of cells with proliferative function and partial differentiation, and their secondary mutations may increase, which may lead to tumorigenesis. If we can learn more about how stem cells are selected between differentiation and renewal, it will help to increase the number of stem cells and maintain their main characteristics, which is also a key step for transplantation and gene therapy by using stem cell potential.

3.3.1 Stem Cell Niches

Stem Cell Niche have the ability to self-replicate, and under certain conditions, they can differentiate into various functional cells. The directed differentiation of stem cells requires a relatively fixed, regulated microenvironment and tissue, which is called the stem cell niche [4]. Stem cell niches are composed of tissue cells and extracellular matrix which contain one or more stem cells and control the self-renewal of stem cells and the production of progeny cells [5]. It allows the distance between cells to be adapted to the interaction between cells and the production and transmission of short-range regulatory factors. Stem cells must proliferate in the niches to maintain self-renewal properties [6]. Adult stem cells are present in a special microenvironment in tissues and are strictly protected by structures such as surrounding cells and matrix [7].

Stem cells are present in the niches [8]. Once required physiologically or pathologically, surrounding cells or factors will send mobilization signals to stem cells to proliferate and differentiate, rapidly replenishing or repairing damaged tissue. Since it is difficult to manipulate stem cells and their surroundings in vivo, currently it is not possible to explicitly study the structure and function of any stem cell niches. In recent years, stem cell niches such as gonads, blood, skin, midgut, nerves, and kidneys have become hotspots in stem cell research [9–11]. A clearer study is the functional stem cell microenvironment of drosophila reproductive stem cells.

3.3.2 Drosophila Reproductive Stem Cell Niches

The gonads of drosophila have been used as a model system for studying stem cell niches, as the drosophila gonads have many advantages in the study of stem cell niches. First, the gonads of drosophila are relatively simple, and their structure has been studied clearly. Secondly, the drosophila gonads contain a single activated germ cell, and their supporting cells are accurately determined. Finally, drosophila germline stem cells (GSCs) produce two progeny cells by asymmetric division. One keeps stem cell characteristics in the stem cell niche, and the other leaves the niche and begins to differentiate.

3.3.2.1 Structure and Characteristics of Drosophila Reproductive Stem Cell Niches

Structure of Drosophila Ovary Stem Cell Niches

The ovary of drosophila is a tubular structure, and reproductive stem cells and their niches are located at the blind end of the apex of the tubular structure. The drosophila ovary tube is a functional unit of ovary containing two to three reproductive stem cells at the apex of the ovary tube. Drosophila has 16 ovary tubes, so the production capacity of them is amazing. The front end of the drosophila ovary contains three different types of cells: terminal filament cells, cap cells, and inner germinal sheath cells (IGSs). Drosophila ovary stem cell niches are composed of these cells [12]. In addition, there is another stem cell, somatic stem cells (SSCs) in the niche [13].

The Niche Structure of GSCs in Drosophila Testis

The testis of drosophila is also a tubular structure [14]. In the testis of adult drosophila, hub cells, germ cells, and proto-encapsulated cells constitute the niches of testis stem cells. Among them, SSCs are in contact with hub cells and are also involved in the formation of niches. The testis stem cells divide in a similar manner to the ovary and in an asymmetric manner. The hub cells in the niches play a major role in signaling pathway. There exist two kinds of stem cells in the drosophila testis niches, namely GSCs and SSCs, just like ovarian.

3.3.3 Stem Cell Niches in Mammalian Tissues

Stem cells are a class of undifferentiated cells or primitive cells that have self-renewal and high proliferative capacity, and can differentiate into at least one mature cell in the body, which is one of the key factors for maintaining the morphology, function, and internal balance of tissues and organs. In recent years, with the development of stem cell research, people have paid more and more attention to the influence of living environment (niches) of stem cells in organism. The microenvironment around the stem cells (also known as stem cell niches or stem cell nests) maintains the characteristics of stem cells, the number of stem cells, and the fate of stem cells by direct and/or indirect interaction between their components (niche cells, extracellular matrix, related cytokines, and signal transduction molecules) and stem cells. In this chapter, we describe the structure of mammalian stem cell niches and their effects on stem cell self-renewal and biological characteristics by taking stem cell niches in germ cell lines, hematopoietic cell lines, epidermis, and small intestinal epithelium as examples.

3.3.3.1 Testicular Germ Stem Cell Niches

Testicular Germ Stem Cells and Their Niches

The testis is an organ that produces sperm and secretes androgen [15]. The surface of the testis is covered with a serosa, and the inner membrane is a white membrane composed of dense connective tissue. The white membrane is thickened at the posterior edge of the testis to form the mediastinum testis, and radiated into the testis parenchyma. The testis is divided into about 250 conical lobules, each containing 1–4 seminiferous tubules. The seminiferous tubules are composed of spermatophytic epithelium. The spermatogenic epithelium is composed of spermatophytic cells and Sertoli cells, which are sites of spermatogenesis in the testis. At the fourth week during embryogenesis, there exist many large round cells derived from endoderm located near the posterior wall of the yolk sac, called primord germ cells. Primordial germ cells migrate to the gonadal ridge in the sixth week and enter the primary sex cord. Therefore, the seminiferous tubules in the embryonic period are solid cell cords, which contain two types of cells, namely, Sertoli cells differentiated from primary sex cord and spermatogonium differentiated from primord germ cells. In adults, the order of the seminiferous tubules from the basement membrane to the lumen is spermatogonia or spermatogonial stem cells (SSC), primary spermatocytes, secondary spermatocytes, spermatoblast, and spermatozoon.

The Sertoli cells mainly support and nourish the spermatogenic cells and promote the production of sperm and the movement of spermatogenic cells to the surface of the seminiferous tubules. Adjacent Sertoli cells are closely connected on the side near basal membrane to divide the spermatogenic epithelium into two parts: the basement compartment and the abluminal compartment. There are spermatogonia in the basement compartment, so the number of Sertoli cells limits the expansion of the number of spermatogonia. At the same time, the Sertoli cells, the basement membrane closely connected with spermatogenic epithelium and myoid cells, constitute the microenvironment (testis germ stem cell niches) for the growth of the testis germ stem cells (Fig. 3.1). The biological behavior of spermatogonial stem cells in the niche is affected by both their own factors and the niche microenvironment. They are slowly renewed or accelerated to keep their population constant, and at the same time produce a large number of differentiated spermatogonia to maintain organ function.

The Role of Testis Germ Stem Cell Niches

Testis germ cell niches provide a specific developmental environment for the self-renewal and survival of spermatogonia. Stem cells in the niche have strong ability to divide and proliferate, and maintain their undifferentiated state.

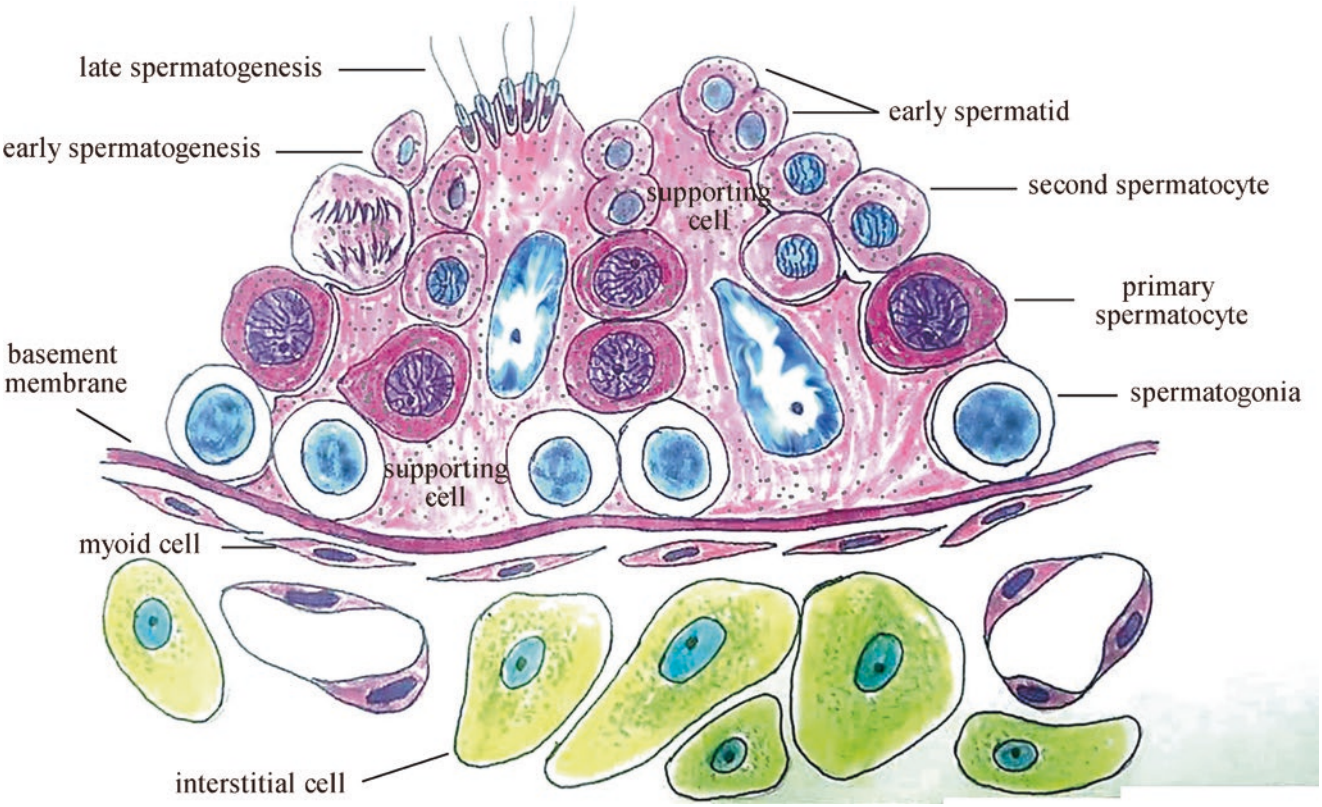

Fig. 3.1 Schematic diagram of testis germ stem cell niche

Studies have shown that when spermatogonial stem cells were transplanted into the seminiferous tubule epithelium of immunodeficient mice, these exogenous germ stem cells could cross the tight junctions of multiple spermatogenic cells and Sertoli cells, search and migrate to the stem cell niches of basal compartment, form progeny clones, and perform biological functions [16].

Related Cytokines in Testis Germ Stem Cell Niches

On the one hand, the biological characteristics of stem cells are the results of their own pre-programming; on the other hand, they are affected by the microenvironment, that is, niches. Niches not only provide a specific developmental environment for stem cell self-renewal and survival, but also shield other factors that induce stem cell differentiation and help stem cells maintain their undifferentiated naive state. Current studies have shown that factors closely related to the differentiation and phenotypic maintenance of testis germ stem cells include c-Kit receptor and its ligand SCF, leukemia inhibitory factor (LIF), vitamin A (vitamin A, VA), epidermal growth factor (EGF), and glial cell line-derived neurotrophic factor (GDNF) [17–21].

3.3.3.2 Bone Marrow Hematopoietic Stem Cell Niches

Location and Concept of Bone Marrow Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) occur very early. Embryonic stem cells produced by several divisions of fertilized eggs begin to differentiate into hematopoietic stem cells while differentiating into embryo and extraembryonic structures. In the extraembryonic structure, hematopoietic stem cells are first seen in the extra-embryonic yolk sac blood island, which is a cell plexus formed by the primordial germ cells (PGCs) of the extraembryonic mesoderm on the yolk sac wall. The outer cells differentiate into vascular endothelial cells, which gradually become longer and connect to each other to form the original vascular network. The inner cells of the blood island remain round, free from the developing primitive vascular network, and differentiate into the earliest blood stem cells. During 16–19 days of gestational period, there are blood stem cells in the extra-embryonic tissues such as the yolk sac wall, body stalk, and chorion, and some stem cells develop into red blood cells. This period is

called the hemopoietic period of yolk sac. During this period, the establishment of blood circulation inside and outside the embryo provides conditions for cell migration. The primordial germ cells present at the base of the extraembryonic allantoic sac begin to migrate to the para-aortic splanchnopleura (PAS) of the embryo and it further developed the aorta-gonad-mesoneph site (AGM), part of which is differentiated into blood stem cells in PAS/AGM. The blood stem cells formed in the embryo migrate to the liver primordium, making the liver the main hematopoietic organ in the fetal period, and also migrate to the spleen, thymus, and bone marrow, providing the most primitive blood stem cells for the formation of hematopoietic organs during fetal development. The yolk sac hematopoiesis terminates at the sixth week of embryonic development and is replaced by fetal liver hematopoiesis into the liver hematopoietic phase. In the 3- to 5-month-old fetal liver, there are about 50% immature red blood cells, only a small number of immature granulocytes and megakaryocytes. During this period, lymphocytes develop mainly in the thymus, spleen, and lymph nodes. Liver hematopoiesis begins to decline at the sixth month of embryonic development and ends at birth, replaced by bone marrow hematopoiesis. The bone marrow is a lifelong hematopoietic organ.

Hematopoietic stem cells have a high degree of self-renewal. Its basic characteristics are as follows:

(a) It has a strong proliferative potential, and can repeatedly divide under certain conditions and proliferate in large quantities, but under normal physiological conditions, most cells are in the G0 quiescent state.
(b) It has a multidirectional differentiation potential. Under the influence of some factors, it can differentiate into different progenitor cells.
(c) It has the ability of self-replication, that is, the daughter cells after cell division still have the original characteristics, so hematopoietic stem cells can maintain a constant amount for life. In hematopoietic tissue, hematopoietic stem cells are difficult to distinguish from other monocytes in morphology. In recent years, due to advances in monoclonal antibody technology, the application of flow cytometric sorting (FACS), research on the surface markers of hematopoietic stem cells has made great progress. It is currently believed that the surface markers of human hematopoietic stem cells are $CD34^+$, $CD38^-$, HLA^-, DR^-, Lin^-, Thy-1^+, c-Kit^+, Sca-1^+, LFA-1^-, $CD45RA^-$, $CD71^-$, Rhodull, and so on.

Bone Marrow Hematopoietic Stem Cell Niches and their Effects

During physiological development, blood stem cells are mainly distributed in hematopoietic tissues. The hematopoietic inductive microenvironment is extremely important, which is the internal environment for growth and development of hematopoietic cells. The hematopoietic inductive microenvironment is a connective tissue composed of bone marrow nerve components, microvasculature system, fibers, matrix, and stromal cells. It is a "niche" for blood stem cells to maintain the best functional state (Fig. 3.2). Stromal cell is an important component in hematopoietic stem cell niches, including reticular cells, fibroblasts, sinusoidal endothelial cells, macrophages, and adipocytes. Bone marrow stromal cells not only support hematopoietic cells, but also secrete cytokines and regulate the proliferation and differentiation of hematopoietic cells. The distribution of various blood cells in the hematopoietic tissue during development has certain regularity. Immature red blood cells are often located near the sinusoids, and they are embedded in the surface of macrophages to form the erythroblastic islet (Fig. 3.3). As the cells mature, they are close to and pass through the sinusoidal endothelium, and their nuclei are removed to become reticulocytes. The immature granulocytes are far away from the sinusoids. When they develop into metamyelocytes and have the ability to move, they approach and penetrate the sinusoids by their amoeboid movement. The megakaryocytes are often attached to the endothelial space of the blood sinusoid, and extend the cytoplasmic processes into the sinus cavity to form platelets after falling off. This distribution indicates that different parts of the hematopoietic stem cell niche have different induction and regulation effects on hematopoietic cells.

Related Regulatory Factors in the Bone Marrow Hematopoietic Stem Cell Niche

Blood circulation ensures the formation of embryonic and fetal hematopoietic organs and the evolution of multistage hematopoietic centers. Similarly, after hematopoietic stem cell transplantation, exogenous hematopoietic stem/progenitor cells migrate, recognize, and locate from peripheral blood to bone marrow through complex molecular interaction. This multistep process is called homing. Hematopoietic stem cells cannot develop normally in the peripheral blood circulation or non-hematopoietic organs. Only after they return to a certain position of the hematopoietic organs through blood circulation, that is, homing, can they exercise their physiological functions. The Wnt signaling pathway, the Notch signaling pathway, and the homeobox (HOX) transcription factor family have all been proven to be closely related to the development and renewal of hematopoietic stem cells, regulating the behavior of hematopoietic stem cells from different levels [22–24].

The self-renewal capacity of HSC is limited, and continuous transplantation can eventually lead to exhaustion of HSC. The senescence of human adult cells is associated with the lack of expression of telomerase and shortening of telomere length. The low level of telomerase activity in

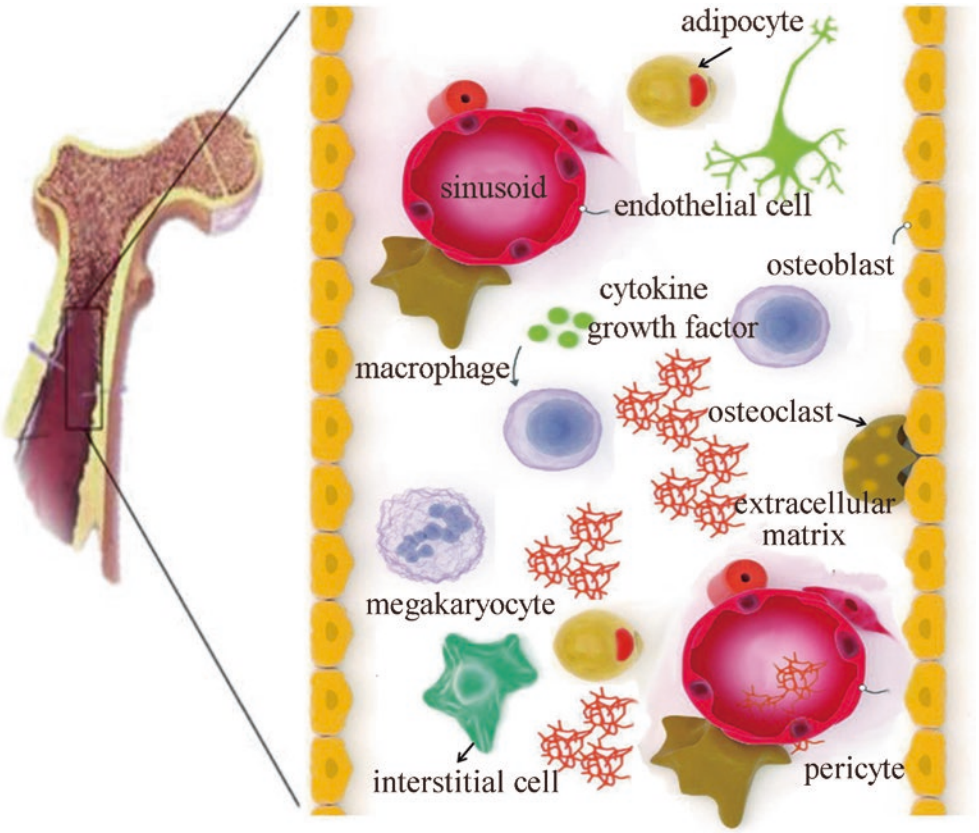

Fig. 3.2 Schematic diagram of the hematopoietic inductive microenvironment of the red bone marrow

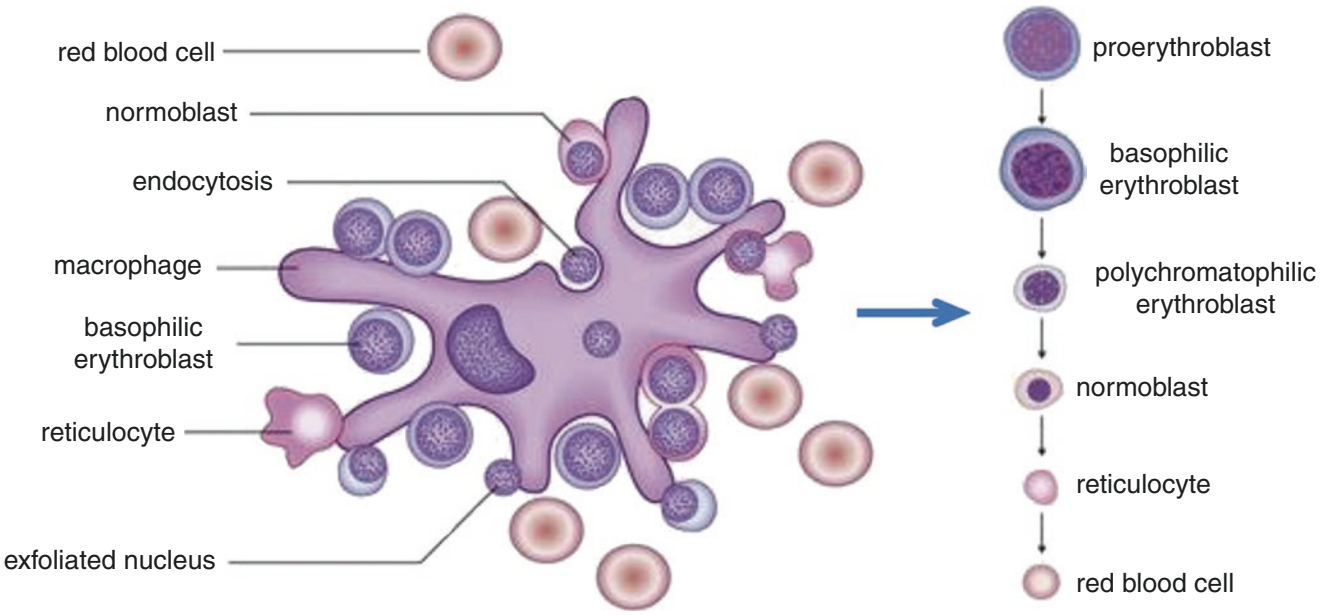

Fig. 3.3 Ultrastructural diagram of bone marrow erythroblastic islet

HSC is not sufficient to inhibit HSC telomere shortening, so it is speculated that HSC overexpression of telomerase or its components can enhance HSC self-renewal ability. However, Allsopp et al. found that telomerase reverse transcriptase (TERT) overexpression inhibited the shortening of telomere length of HSC in continuous transplantation, but the hematopoietic reconstitution ability of HSC in transgenic mouse was not different from that of wild type [25]. Overexpression of the antiapoptotic gene bcl-2 in HSC can increase the number of HSCs, indicating that anti-

apoptotic signals are involved in the regulation of HSC self-renewal. Cyclin-dependent kinase inhibitor (CKI) p21 controls stem cells into the cell cycle and is an important factor in maintaining HSC in quiescence.

In the absence of HSC, activation into the cell cycle and self-renewal ability are impaired. P18 specifically acts on the early G1 of the cell cycle and binds to cyclin-dependent kinase (CDK) 4/6 to inhibit phosphorylation of retinoblastoma protein (Rb) and arrest cell cycle progression [26]. At present, the relationship between Wnt signaling pathway, Notch signaling pathway, and HOX transcription factor family regulating HSC self-renewal and CKI is still unclear, and further research is needed [27].

3.3.3.3 Skin Stem Cell Niches

Location and Concept of Skin Stem Cells

Skin stem cells, that is, keratinocyte stem cells, are mainly located in the basal layer of the epidermis, the periphery of the sebaceous glands, and the bulge of root sheath outside the hair follicle [28]. Skin stem cells self-renew through asymmetric division, and at the same time form a new daughter cell, transit-amplifying cells (TA cells) [29]. The transit-amplifying cells continue to divide, expand, and move up to form other cell layers such as granular cell layer, prickle cell layer, and the like. With the passage of time, the granular cells and spinous layer cells mature and perform the tissue function, gradually aging and losing their organelles, which are replaced by the keratin secreted by the cells themselves to form a transparent layer or a stratum corneum until falling off. This process can be called anagen, catagen, and telogen. The epidermis is following this continually recurrent physiological process, self-renewal, and self-healing.

Like other stem cells, keratinocytes have the following characteristics: the cell morphology is naive, maintaining a relatively undifferentiated state; it has strong self-renewal ability; in normal state, the cell cycle of stem cells is slow, but it can also respond to external stimuli, rapidly enter cell division cycle, and maintain the integrity of organ or tissue morphology and function. Potten used 3H-thymidine absorption method to measure skin stem cells. It was found that the cells with slow metabolism of 3H-thymidine were mainly located in the basal layer of the epidermis, while the cells with active metabolism were located in the suprabasal layer. Jones and Watt found that epidermal stem cells can express integrins β1. Later, Kaur study found that keratinocytes can also express integrins α6. The cells with strong positive expression of these integrins β1 and α6 not only showed strong proliferative ability, but also showed different adhesion ability to extracellular matrix. In 1980, Fuchs and Green reported for the first time that skin stem cells can express keratin 19 (K19) [30].

There are many types of keratin, which are widely expressed in skin tissues. The occurrence and development of skin are closely related to the programmed expression of keratin gene. K5 and K14 are mainly expressed in the basal layer and upper basal layer of the primary epithelium of the embryonic mouse. After 9.5 days of embryonic development, K5 and K14 are only expressed in the basal layer of the epidermis and downregulated. K1 and K10 are mainly expressed in the granular cells and spinous layer cells. In the process of studying the expression of cytokeratin in skin, Li Haihong found that K7 is mainly expressed in the outer root sheath of hair follicles in fetal period, and has no obvious expression in adult stage. K7 can be used as an indicator to study the development and maturity of hair follicles. K10 is mainly expressed in the basal layer, which can be used as a marker for terminal differentiation of epidermal cells. K14 is strongly expressed in the basal layer and skin appendages. Bokis Risek found that the expression time, expression level, and expression site of the gap junction gene-encoding products α1 and β2 connexin are related to the directional differentiation of skin stem cells. The intermediate filament protein Nestin, which is abundantly expressed in neuroepithelial cells in early embryonic development, can also be strongly expressed in hair follicle precursor cells. In 1996, Thierry Magnaldo reported that CD24 cDNA is abundantly expressed in the cytoplasm of keratinocyte colony-forming cells (K-CFCs), which are mainly distributed in the bulge, one-third of outer root sheath and the end of the hair ball. As the hair follicle matures, CD24 cDNA expression also showed relative displacement changes. Although CD24 does not directly act on the self-renewal of keratinocytes, it provides a reference for distinguishing keratinocytes from differentiated cells at the molecular level. In 2000, Hiroaki successfully isolated stem cells from TA cells by using in vivo cell dynamic analysis and fluorescent living cell tracer technology and demonstrated that mouse epidermal stem cells have high-level expression of α6 integrin and low-level expression of transferrin receptor (CD71), so it is also called α6bri/CD71dim cells; the TA cells are positive for α6 integrin and CD71, so it is called α6bri/ CD71bri cells.

Skin Stem Cell Niches and their Effects

Skin stem cells play an important role in maintaining the balance, renewal, and repair of the internal environment of tissues and organs. Under normal circumstances, skin stem cells proliferate and differentiate according to a certain probability and mode. When the body is damaged, the proliferation and differentiation mode of skin stem cells will change to meet the needs of the body. Therefore, the proliferation and differentiation behavior of skin stem cells are preprogrammed on the one hand; on the other hand, they are regulated by the microenvironment of skin stem cells (also known as skin stem cell niche). Nowadays, more and more studies

have shown that hair follicle bugle is one of the concentrated areas of skin stem cells, where cells not only have strong proliferative capacity, but also have the potential of multidirectional differentiation, so bulge is also known as hair follicle stem cell niche (Fig. 3.4). In 1990, Cotsarelis first proposed the "bulge activation hypothesis" (Fig. 3.5). Subsequently, Taylor and Oshima confirmed the localization of hair follicle stem cells through experiments, and proposed the "hair follicle stem cell migration hypothesis." The hypothesis is that during the periodic growth of hair follicles, the cells in the bugle migrate down to the hair ball after being induced by some factors, transform into proliferative hair matrix cells, and then proliferate and differentiate into hairs upward and inward. When the cells in bugle receive some unknown signal and stop producing new stem cell individuals, the growth phase ends. In 2000, Commo et al. proposed the "hair follicle stem cell reservoir hypothesis" by studying K19 staining in the outer root sheath of hair follicles. They speculated that the outer root sheath of the hair follicle contained two stem cell regions, one migrated down with the newborn hair follicle and the other was fixed at the bulge of the hair follicle. Although there is still much controversy in this hypothesis, it explains the origin of trichoepithelioma and the reasons for the growth and regeneration ability of the hair follicles after being removed.

The stromal cells surrounding the dermal papilla in the decline period stop dividing and expanding, the hair follicles

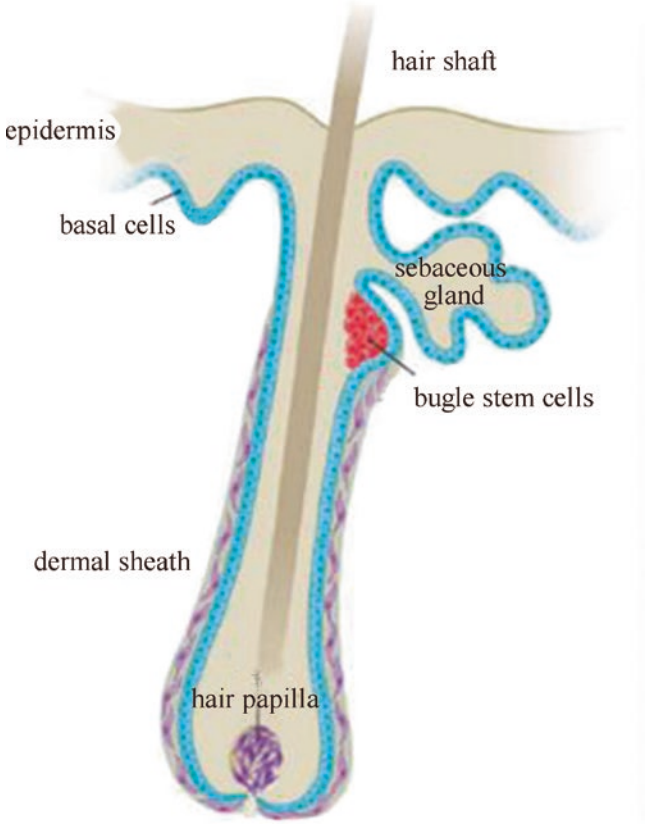

Fig. 3.4 Schematic diagram of hair follicle stem cell niche

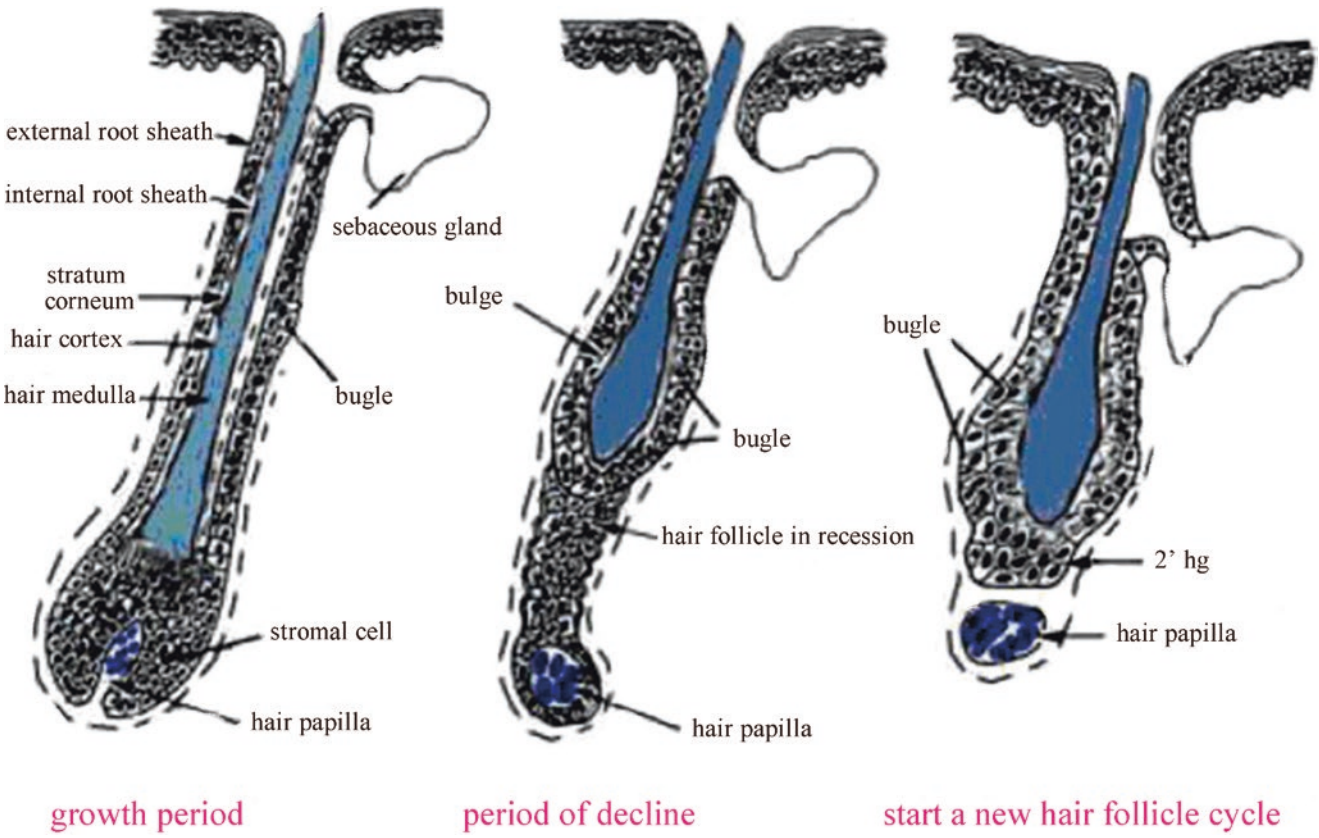

Fig. 3.5 Bulge activation hypothesis

no longer grow, and the bottom is degenerated; then the DP cells move up to the outer root sheath to enter the stationary phase; the dermal papilla interacts with the bulge cells to induce stromal cells and outer root sheath cells to divide, expand, and move down, forming a concentric circle-like IRS and hair root (growth stage) according to a certain spatial structure.

Skin stem cell niches regulate the proliferation and differentiation of skin stem cells mainly through cells and cells, cells and extracellular matrix [7]. Cytokines play an important role in the transmission of information between cells and extracellular matrix, cells and cells [31, 32]. Extracellular matrix components such as fibronectin, laminin, and different types of collagen are involved in the maintenance of epidermal stem cell characteristics and the regulation of biological processes such as proliferation and differentiation. The gap junction between the epidermal stem cells, as one of the information channels between niches and the outside world, limits the differentiation direction of the epidermal stem cell community. In addition, recent studies have shown that many signal transduction-related factors are involved in the regulation of keratinocyte self-renewal, division, and proliferation, such as lymphoid enhancer factor/T cell factor (LEF/TCF), tumor necrosis factor (TNF), transcription factor Myc, and T lymphocyte differentiation regulator GATA-3.

Skin Stem Cell Niche-Related Signaling Regulators

At present, it has been found that the signaling pathways that maintain and regulate epidermal stem cell status are mainly MAPK, Wnt, Notch, BMP, and Shh. In addition, there are many other signal pathways to be discovered.

(a) Mitogen-activated protein kinase (MAPK) [33]: MAPK is a class of protein kinase with serine/threonine residues, which is widely distributed in cells and a group of important signal-regulating enzymes that are closely related to membrane surface receptors and decisive genes expression. The MAPK pathway is an important nuclear activation transcription factor response pathway involved in the regulation of various physiological and pathological processes such as growth, differentiation, division, death, and synchronization of cell functions.
(b) Wnt signaling pathway [34]: Wnt gene encodes a variety of secretory signaling molecules, which are related to the occurrence and development of many tissues and organs, such as brain, nerve, spinal cord, bone, retina, and kidney. A variety of Wnt proteins such as Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt7b, Wnt10a, Wnt10b, Wnt11, and Wnt16 can be expressed in the skin. Wnt10a and Wnt10b are expressed in the primordial primordium of hair follicles, and Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt10a, Wnt10b, and Wnt11 are expressed in hair follicle anterior cortex cells.
(c) Notch signaling pathway [35]: The Notch signal transduction pathway regulates the fate specification of embryonic and terminally differentiated tissue cells through local cell-cell interactions, and is mainly involved in promoting the differentiation of root sheath keratinocytes in hair follicles.
(d) Bmp signal transduction pathway [36]: Bone morphogenetic protein-4 (BMP-4) can induce the expression of LEF1 in mesenchymal cells at the early stage of hair follicle primordium formation. Bone morphogenetic protein and bone morphogenetic protein inhibitors regulate the expression of LEF1 by regulating the homeostasis between them, thereby determining the response of mesenchymal tissue cells, epithelial precursor cells, and hair follicle progenitor cells to Wnt signaling pathway.
(e) Shh signal transduction pathway: Shh is mainly expressed in stromal cells at the end of hair follicles during the growth phase and is involved in the regulation of hair follicle stem cell migration and differentiation during hair follicle growth and regeneration. When the Shh function is blocked, the hair follicles stop growing, and the ectopic expression of the Shh target gene can cause the occurrence of hair follicle tumors.

3.3.3.4 Intestinal Stem Cell Niches

Intestinal Stem Cells and Their Niches

Mammalian intestinal mucosal epithelium is the most active place of the body's metabolism. The intestinal mucosal epithelial cells undergo continuous self-renewal throughout the life, which is mainly driven by pluripotent stem cells located at the bottom of the small intestine crypt. The stem cells located at the bottom of the small intestine crypt maintain a strong proliferation and differentiation ability [37], and there is a dynamic balance between the cells with apoptosis and damage necrosis and stem cell with proliferation and differentiation. Therefore, intestinal stem cells play an important role in maintaining the structural and functional integrity of the intestinal barrier and repairing after injury. The epithelium of the small intestine consists of monolayer columnar cells, such as absorptive cells, goblet cells, M cells, Paneth cells, and a small number of endocrine cells. The intestinal epithelium of human, mouse, and rat can be functionally divided into two discrete units: the villus differentiation unit and the crypt proliferation unit. The small intestinal stem cells proliferate and differentiate to form oriented progenitor cells, and the oriented progenitor cells rapidly differentiate into Paneth cells, goblet cells, M cells, absorptive cells, and endocrine cells. Paneth cells migrate down to the bottom of the crypt, and the remaining four cells migrate toward the intestinal lumen to supplement the mucosal epithelial cells

which shed from the top of the villi. The entire migration process takes 3–5 days. The main characteristics of small intestinal stem cells are as follows:

(a) Stem cell markers are relatively clear and can express surface marker proteins such as musashi-1, Sca-1, keratin-6, integrinβ1, Hes1.
(b) With radiation hypersensitivity, small dose irradiation can induce apoptosis.
(c) Small intestinal stem cells selectively isolate chromosomes to retain the characteristics of the parental stem cells and maintain the stability of the genetic material.
(d) Small intestine crypts and adjacent villi in adult are monoclonal sources.

In addition, the proliferation and differentiation of small intestinal stem cells is achieved by the division of crypts. The small intestinal stem cells are located in the fourth to fifth layer from the bottom of the small intestine crypt, above the Paneth cells. Each crypt has 4 to 6 actual stem cells and about 30 potential stem cells. Small intestinal stem cells maintain a stable number of stem cells in the crypt by symmetric and asymmetric divisions. Which way is the main one depends on the internal environment in which the stem cells are located. Under normal physiological conditions, small intestinal stem cells of adult mice generally undergo proliferation and differentiation processes by asymmetric division. Therefore, supporting cells, extracellular matrix, and secretory cytokines derived from supporting cells in the small intestine crypt constitute stem cell niches that affect the proliferation and differentiation behavior of small intestinal stem cells (Fig. 3.6).

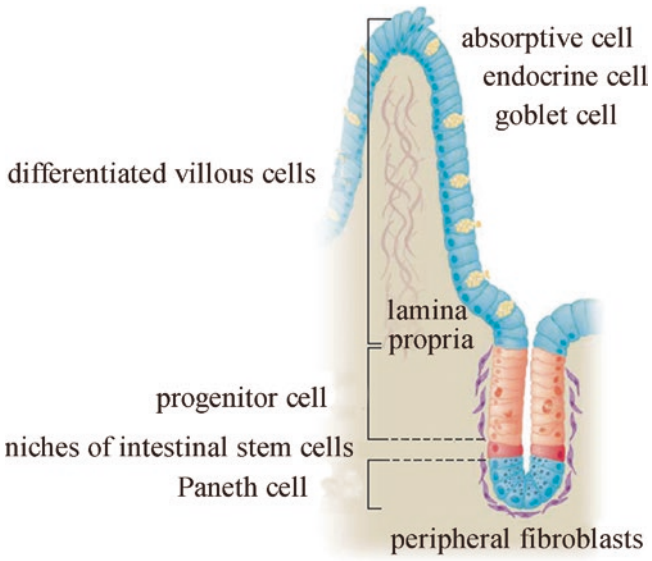

Fig. 3.6 Schematic diagram of intestinal stem cell niche

As shown in the figure, the small intestine stem cells are located in the fourth to fifth cells from the bottom of the small intestine crypt, above the Paneth cells; the supporting cells, the extracellular matrix, and the secretory cytokines derived from the supporting cells in the small intestine crypt constitute microenvironment for the proliferation and differentiation of small intestinal stem cells.

Intestinal Stem Cell Niche-Associated Regulatory Molecules

Small intestinal stem cell niches provide a material basis for the proliferation and differentiation of intestinal stem cells, in which cytokines play an important role in affecting the proliferation and differentiation of stem cells, such as basic fibroblast growth factor (bFGF) [38], transforming growth factor-β (TGF-β), EGF, TNF, IGF-1, and the like [39]. In addition, many apoptosis-related genes such as p53 [40], Bcl-2 [41], p38, c-Fos, and c-Jun have also played an important regulatory role in the proliferation and differentiation of intestinal stem cells.

Intestinal Stem Cell Niche-Related Signal Transduction Pathway

As for the effects of small intestinal microenvironmental signal transduction on stem cell maintenance and differentiation, the current research focuses on the Notch and Wnt/β-catenin signaling pathways; the former determines the fate of stem cells differentiation, and the latter determines the maintenance of stem cell status [1, 42].

3.3.3.5 Neural Stem Cell Niches

Neural Stem Cells and Their Niches

In 1992, Reynolds et al. isolated stem cells from the corpus striatum of rat brain, which can continuously proliferate in vitro and have potential to differentiate into neurons and astrocytes [43]. Since then, the isolation, culture, identification, and research in vitro of neural stem cells have made gratifying progress. Neural stem cells (NSCs) are specific primitive nerve cells in the nervous system that can proliferate and differentiate into neurons and glial cells. They have the characteristics of high self-renewal ability, multipotential differentiation, migration function, and good tissue fusion [44]. NSCs are widely distributed in the central nervous system of mammalian embryos, including the cerebral cortex, corpus striatum, hippocampus, olfactory bulb, cerebellum, spinal cord, etc. In adult, NSCs are limited to the hippocampal dentate gyrus and subependymal zone. At present, the specific surface marker proteins of NSCs are still not well defined, and Nerns is commonly used as a detection marker to identify NSCs [45]. Nestin belongs to the class VI intermediate filament protein and is abundantly expressed in

the neuroepithelial cells in the early stage of embryonic development. However, in recent years, it has been found that Nestin is not only expressed in neural stem cells, but also expressed in many tissues, such as pancreatic precursor cells, hair follicle precursor cells, human umbilical cord blood mononuclear cells, bone marrow stromal cells, pancreatic vascular endothelial cells, etc. Other neural stem cell markers such as Vimentin, RNA binding protein Musashi-1, CD133, glial fibrillary acidic protein (GFAP), etc. are less used due to relative lack of specificity.

The proliferation and differentiation of neural stem cells are affected by the surrounding microenvironment [46, 47]. The state and the microenvironment of the stem cells determine whether the stem cells are in the resting or dormant state, whether they differentiate or mature, and which type of cells they are going to differentiate [3]. For example, in mammals, only the neural stem cells of the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus can differentiate into neuronal cells in vivo, while in other regions neural stem cells can only differentiate into glial cells in vivo. If the stem cells of the neurogenesis area are transplanted into a non-neurogenic region, these stem cells can only differentiate into glial cells, and stem cells derived from non-neurogenic regions can differentiate into neurons after being transplanted into the neurogenesis region [48]. The microenvironment surrounding the neural stem cells is also called neural stem cell niche or neural stem cell nest, which mainly includes nearby supporting cells, extracellular matrix, microvascular network, cell regulatory factors, and neurotransmitter mediators. These components interact to form a complex regulatory network that affects the biological characteristics and behavioral outcomes of neural stem cells.

Neural Stem Cell Niche-Related Regulatory Molecules

The effects of neural stem cell niches on the differentiation of NSCs are mainly reflected in the following four aspects:

(a) The effect of cytokines on the differentiation of NSCs: Cytokines play a key role in the growth and differentiation of NSCs. Currently, growth factors can be used to separate NSCs from brain tissue, which can make stem cells further proliferate and differentiate in vitro, so as to provide a large number of seed cells for the study of biological characteristics of stem cells and stem cell transplantation. Among them, FGF and EGF are widely studied.

In addition, in vitro experiments have also observed that brain-derived growth factor (BDNF), insulin-like growth factor-1 (IGF-1), and retinoic acid can promote the differentiation of NSCs into neuron phenotypes. The neurotrophic factor NGF has the ability to induce the differentiation of NSCs into cholinergic neurons and promote the growth and thickening of differentiated neuronal processes. Platelet-derived growth factor (PDGF) and retinoic acid can induce hippocampal NSCs to differentiate into neurons.

(b) The effect of intercellular interaction on NSCs differentiation: The supporting cells of the neural stem cell nest mainly include astrocytes, ependymal cells, and radial neuroglia cells. When NSCs are in different cellular environments, their differentiations are not the same. Intercellular interactions affect NSC differentiation and maturation [49].

(c) The effect of extracellular matrix on neural stem cells: Extracellular matrix (ECM) is composed of various glycoproteins, mucins, neuroadhesion factors, etc., which is an important part of neural stem cell nest in space. ECM can play the role of microenvironment signal amplifier by adjusting adhesion and signal molecule aggregation [50].

(d) The effect of Notch signal transduction pathway on NSCs differentiation [2]: Although many foreign signals play an important role in stem cell development and differentiation, it does not negate the role of stem cell expression in regulating differentiation and development. There is evidence that the diversity of neural cell development may be related to the expression of various transcription factors in stem cells, and the expression of different transcription factors leads to the differentiation of different lineages. Gene regulation of NSCs involves both positive and negative dual regulation. Negative regulation keeps NSCs in their undifferentiated state, increasing the number of NSCs by symmetric division, mainly including Notch signaling pathway; positive regulation promotes NSCs differentiation by asymmetric division, and bHLH (basic helix loop helix) transcription factors include Mash1, NeuroD, Neurogenins (Ngn1/Ngn2), and the Mash family. The Notch signaling pathway, which conducts the Mash signal of interaction between cells, precisely regulates the behavior of cells in each lineage through signal transmission between adjacent cells and plays a key role in cell differentiation [51].

3.3.4 Summary

Stem cells are a class of undifferentiated primitive cells with strong proliferative capacity and multidirectional differentiation potential [52]. On the one hand, the biological characteristics of stem cells are the results of self-preprogramming; on the other hand, they are affected by the microenvironment. Stem cell niches can exert multiple biological effects through direct and/or indirect effects between its components (niche cells, ECM, and the cytokines secreted by niche

cells) and stem cells. The functions of stem cell niches are summarized below.

(a) Anchoring stem cells and regulating their presence in the G0 phase: This ability of stem cell niches is related to the expression of adhesion molecules by their components. Cadherin- and integrin-mediated cell adhesion are ubiquitous. The mechanism of anchoring stem cells in niches is that the integrin on the surface of stem cells can mediate the adhesion between stem cells and ECM, so that stem cells can settle in the niches. In addition, stem cell niches can also recruit new stem cells and make them migrate back into the niches, which are the "homing" phenomenon of stem cells.
(b) Maintaining the balance between self-renewal and differentiation of stem cells: Stem cell function is dual. It is necessary to maintain the self-renewal of stem cells as well as the differentiation of stem cells. Stem cells generally self-renew through asymmetric division and produce daughter cells at the same time; or maintain the balance between self-renewal and differentiation by alternate symmetrical proliferation and differentiation. The niche structure is asymmetrical, and this asymmetry may be combined with the mechanism of stem cell division to determine the division mode of stem cells and affect the fate of the daughter cells.
(c) Regulating stem cell fate: Stem cell niche provides a protective microenvironment for stem cells, shielding the influence of externally induced differentiation factors on stem cells and maintaining the undifferentiated state of stem cells. Niche components: niche cells, extracellular matrix, and soluble factors derived from niche cells can regulate stem cells by direct or indirect interaction with stem cells. The change of niche signals can cause changes in stem cell fate. A variety of growth factors related to niche, such as interleukin, Wnt protein, EGF, and its family members, IGF, TGF-β, LIF, etc., are closely related to the proliferation and differentiation of stem cells. In addition, multiple signaling pathways such as Wnt, BMP, MAPK, Notch, Shh, and Tie2/Ang-1 interweave into a network to control the behavior of stem cells [53].

At present, stem cells have become a hotspot in life science research, which makes people see the hope of conquering diabetes, Parkinson's disease, tumor, and other clinical diseases that are still difficult to cure. With the further study of stem cells and their surrounding microenvironment, people can control the self-renewal and differentiation of stem cells through regulation of stem cell niches, which can provide new research strategies for the treatment of tumors and other degenerative diseases and the development of regenerative medicine.

3.4 Signals Involved in Tissue Repair and Regeneration

Wound repair and tissue regeneration refer to a series of complicated and orderly pathological and physiological processes that local tissues complete repair and reconstruction through regeneration and replacement after tissue loss due to the role of wound causing factors. It is a cascade reaction involving local inflammatory cells, extracellular matrix, damaged site cells, blood-derived cells, growth factors/cytokines, and even nervous system, immune system, and endocrine system. The process mainly includes the inflammatory phase, the proliferative phase, and the remodeling phase. A series of biological activities involve the transmission of cellular signals to complete all biological effects.

3.4.1 Mitogen-Activated Protein Kinase

Mitogen-activated protein kinase (MAPK) is a kind of serine/threonine protein kinase widely distributed in animal cells and plant cells. Its main function is to transmit extracellular stimulation signals into cells and nuclei to cause cellular biological reactions (proliferation, differentiation, stress, apoptosis, etc.). Multiple parallel MAPKs signaling pathways have been identified. Different extracellular stimuli activate different MAPKs signaling pathways to mediate different cellular biological responses. Four different MAPKs pathways have been discovered in mammals: the extracellular signal-regulated kinase (ERK) pathway, the JNK/SAPK pathway, the p38/MAPK pathway, and the ERK5 pathway. These four pathways are activated by independent (sometimes interactive cross-talk) signal cascades.

3.4.1.1 Extracellular Signal-Regulated Kinase

Extracellular signal regulated protein kinases (ERKs) are a class of protein kinases that are distributed in the cytoplasm and have the dual phosphorylation ability of serine and tyrosine. They are important members of the MAPK family. ERK includes five families: ERK1/ERK2, ERK5, ERK3/ERK4, of which ERK1/ ERK2 is the first cloned member of the MAPK family. Mitogen can activate ERK1 and ERK2 in almost all mammalian cells. ERK1 and ERK2, also known as p44MAPK and p42MAPK, have relative molecular masses of 4400 and 4200, respectively, and are downstream signaling proteins of various growth factors (EGF, bFGF, NGF, PDGF, IGF, etc.). The basic signal transmission steps follow the three-stage enzymatic cascade of MAPKs, that is, upstream activating protein → MAPK kinase kinase (MAPKKK) → MAPK kinase (MAPKK) → MAPK. The signaling pathway it mediates involves the core of the signaling network that regulates cell growth, development, and division. ERK can also be phosphorylated (p-ERK) by acti-

vation of ion beam irradiation and hydrogen peroxide, enter the nucleus to act on transcription factors such as c-Myc, AP-1, NF-κB, and promote the transcription and expression of certain genes, which are related to the process of cell death and transformation.

Skin acts as the largest organ of the body, and the maintenance of the structure and function of the epidermal layer is mainly dependent on the basal cells of the epidermis. The cells at this site are composed of epidermal stem cells and transient expansion cells. Stem cells rapidly divide and proliferate, form short-expanded cells, and further differentiate, producing a large number of cells with different structures and functions. During the migration of these cells to the epidermal layer and dermal layer, on the one hand, they have supplemented apoptotic or necrotic cells; on the other hand, they form new structures and tissues that cause thickening of the epidermis and dermis. The transition of stem cells from proliferation to differentiation occurs in the G1 phase of the cell cycle. The conversion of cells from the resting phase to G1 phase requires the involvement of the ERK1/ERK2 signaling pathway. Observation of hypertrophic scar tissue showed a higher rate of positive cells for Ras and phosphorylated ERK1/ERK2. This may be related to the rapid division, proliferation, and differentiation of skin stem cells by activation of ERK1/ERK2 pathway to form terminally differentiated cells with special structure and function, especially fibroblast, which is one of the mechanisms of hypertrophic scar formation and maturation.

3.4.1.2 C-Jun N-Terminal Kinase (JNK) Signaling Pathway

JNK signal transduction pathway is a member of the MAPK superfamily. It is originally named for its phosphorylation of c-Jun (a transcriptional regulator, a member of the leucine zipper family), N-terminal active regions Ser63 and Ser73. JNK signal transduction pathway can be activated by tumor necrosis factor (TNF), epidermal growth factor (EGF), interleukin (IL) and other growth factors/cytokines, and some guanylate-binding proteins (G-protein)-coupled receptors and stress, and participate in cell proliferation and differentiation, cell morphology maintenance, skeleton construction, programmed cell death, cell malignant transformation, and other biological reactions.

The typical JNK signal transduction pathway is activated by the MAP3K, MAP2K, and JNK three-stage enzyme chain reactions. During this process, MAPKK4 and MAPKK7 are double-phosphorylated and activate Thr and Tyr in the characteristic modular structure of Thr-proline-tyrosine. JNK in the cytoplasm moves into the nucleus and activates the phosphorylated substrate of JNK. The regulation mechanism of JNK signal transduction pathway is complex, in addition to phosphorylation regulation; it is also regulated by signal component modularization, ubiquitin, and signal crosstalk.

JNK is a major signal transduction molecule in cells. It can induce apoptosis in stress response after injury and is closely related to cell proliferation. Studies have shown that in hypertrophic scar tissue, p-JNK is present in the basal layer and part of fibroblasts, while in normal skin, p-JNK is localized in the basal layer of the epidermis. Studies even found that p-JNK was expressed by polymorphonuclear granulocytes, monocytes, and fibroblasts in the healing process of mouse skin wounds. p-JNK has a regulatory effect on the apoptosis of these cells and then affects wound healing.

JNK Signal Transduction Pathway Regulates Migration and Repair Process of Epithelial Cells and Fibroblasts

During the process of EGF-induced migration of human corneal epithelial cells, JNK signal transduction pathway is activated, and paxillin (Pax) Ser178, Tyr31, and Tyr118 sites are phosphorylated. Inhibition of RhoA activity or JNK inhibitors delays ActB-induced wound healing. Further experiments confirmed that ActB promotes epithelial wound healing through the RhoA-Rho-related rho-associated coiled-coil protein kinase (ROCK)-JNK-c-Jun signal transduction pathway. Studies have shown that ActB can promote muscle tension fibers formation and bone marrow mesenchymal stem cells migration by activating JNK and ERK signaling pathways, thereby promoting bone marrow mesenchymal stem cells-mediated wound healing.

Studies have shown that the JNK signal transduction pathway is involved in the fibroblast migration process. Retinoic acid-related orphan receptors-2 (ROR-2) associates with filamin A (FLNa) and protein kinase C (PKC) during mouse embryonic fibroblasts migration induced by wingless-type mice mammary tumor virus integration site family (WNT) 5a, then activates JNK at the wound edge to promote cell polarity and migration. However, inhibition of JNK or PKC signaling pathways can inhibit WNT5a-induced JNK activity and microtubule tissue center relocalization. ROR-2 is required during WNT5a-induced cell migration, and the mechanism by which FLNa and PKC regulate JNK activity and how activated JNK in turn regulates cell polarity and migration needs further study. Tan Zhen et al. found that the PI3K-Rac1-JNK signal transduction pathway plays an important role in bFGF regulation of fibroblast migration. Wang et al. found that JNK is involved in WNT5a regulation of adhesion and migration of human dental papilla cells, and that WNT5a may or may not rely on the RhoA signal transduction pathway to activate the JNK pathway. The results suggest that the migration of human dental papilla cells is inhibited after inhibition of the JNK signal transduction pathway, and the mechanism may be related to the inhibition of focal adhesion formation.

JNK Signal Transduction Pathway Regulates Cell Migration Process in Inflammatory Environment

Macrophage migration inhibitory factor (MIF) is a chemokine-like multi-effect pro-inflammatory cytokine. In T-cell and fibroblasts, MIF can trigger the 4-CD74-SRC-PI3K axis of cysteine-X-cysteine chemokine receptor to activate JNK signal transduction pathway rapidly and temporarily upregulate the gene expression of inflammatory factor cysteine-X-cysteine chemokine ligand 8. Catestatin (a neuroendocrine antimicrobial peptide) induces mast cell migration and degranulation, and produces cytokines and chemokines. Catestatin-mediated mast cell activation is regulated by GBP, phospholipase C, and MAPK-ERK signaling pathways. In MAPK signal transduction pathway, both ERK and JNK are phosphorylated (p38 is not activated), and ERK-specific inhibitor U0126 almost completely suppresses the production of cytokines and chemokines stimulated by the catestatin, whereas JNK inhibitor SP600125 does not. The JNK signal transduction pathway is involved in the activation of mast cell mediated by catestatin, but its role is not necessary, and its regulatory mechanism needs further study. A variety of pro-inflammatory stimuli, such as IL-1, IL-6, TNF-α, and lipopolysaccharide, can activate the JNK signaling pathway and regulate cell migration. Studies have shown that TNF-α- and IL-1-induced or transforming growth factor β-induced nuclear factor-κB (NF-κB) inhibits the activation of protein kinase and JNK-AP1 signal transduction pathway.

Xiaobing's Fu team used 18 cases of full-thickness skin on the back of the fetus whose pregnancy was terminated due to unexpected factors such as trauma [54]. The embryo gestational age (EGA) was 13 to 32 weeks, including 2 cases of EGA 13, 15, 17, 21, 23, 25, 28, 30, and 32 weeks, respectively. According to the different periods of fetal development, the fetal skin specimens were divided into three categories: early pregnancy fetal skin (EGA 13–17 weeks), mid-pregnancy fetal skin (EGA 21–25 weeks), and late pregnancy fetal skin (EGA 28–32 weeks). The corresponding six cases of children's skin were normal skin of the back or upper limbs due to plastic surgery, aged 4–12 years. The total RNA from fetal and children skin of different gestational ages was extracted, and the quality and concentration of the extracted total RNA were detected by ultraviolet spectroscopic analysis and gel electrophoresis. The mRNA was isolated and purified according to the PolyATtract mRNA Isolation System, and its purity was analyzed by ultraviolet spectrometry. The RT-PCR products of the JNK-1 and JNK-2 genes were 267 and 308 bp, respectively. The expression level of the JNK-1 gene rises to the highest in the fetal skin of the second trimester and then gradually decreases. During fetal skin development, the expression of JNK-2 gene gradually increased. In children's skin, the gray density of the expressed product was 2.1 times that of early fetal skin, and the gene expression was significantly enhanced ($P < 0.01$). In the skin tissues of early pregnancy, the expression level of JNK3 gene was low, and the expression of this gene was significantly increased in the skin of late pregnancy and children ($P < 0.05$).

3.4.1.3 p38 Mitogen-Activated Protein Kinase

The first discovered p38 MAPK member is p38α, a 38 ku protein whose tyrosine site was rapidly phosphorylated by LPS stimulation. This protein is a target of pyridazoles, which can inhibit the production of inflammatory factors such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) in LPS-stimulated monocytes. In addition, the protein can activate MAPK-activated protein kinase 2 (MK2) when the cells are subjected to heat shock, arsenite, or IL-1. Three other homologs of p38α were also found, namely p38β, p38γ (SAPK3, ERK6), and p38δ (SAPK4). The four members of the p38 family are encoded by different genes and expressed differently in different tissues. P38α and p38β are expressed in almost all tissues, p38γ is mainly present in muscle tissue, and p38δ is mainly expressed in testis, pancreas, and small intestine. The active substrates of these members have partial overlaps, but each also has its own specific substrate.

The biological roles of p38 mitogen-activated protein kinases include cell differentiation, migration, cell cycle regulation, and inflammatory responses. Schematic diagram of the p38MAPK signaling pathway is shown in Fig. 3.7 [55]. In the skin tissue, the experimental results showed that the expression of p-p38MAPK was observed in the epidermal layer, sarcolemma, hair follicle, and sebaceous gland of the normal skin of mice, which was related to the physiological function, distribution, and the maintenance of skin homeostasis of p38MAPK. P38σ regulates the activity of the involucrin during differentiation of keratinocytes. It has been reported that under normal conditions, tyrosine 323 site is autophosphorylated, and p38MAPK expression in T lymphocytes is activated by T cell receptor (TCR), but not in B lymphocytes. In the process of skin damage repair, it mainly divides into inflammatory phase, fibroproliferative phase, and tissue remodeling phase. In these three consecutive processes, neutrophils, monocytes, and fibroblasts play a major role, and signal is involved in cell migration, inflammatory response, and cell cycle regulation. In the early stage of skin injury, neutrophils infiltrate the damaged area, and then neutrophils undergo apoptosis, which is cleared by macrophages. In the past, it was thought that neutrophil apoptosis was related to Fas/FasL and ROS. Other studies have shown that p-p38MAPK is always expressed during neutrophil apoptosis, without activation of ERK and JNK proteins. The apoptotic effect of p38MAPK on neutrophils is not necessarily achieved by activation of Fas/FasL and ROS. At least two pathways mediate neutrophil apoptosis, one is p38MAPK and the other is Fas/FasL, and eventually may

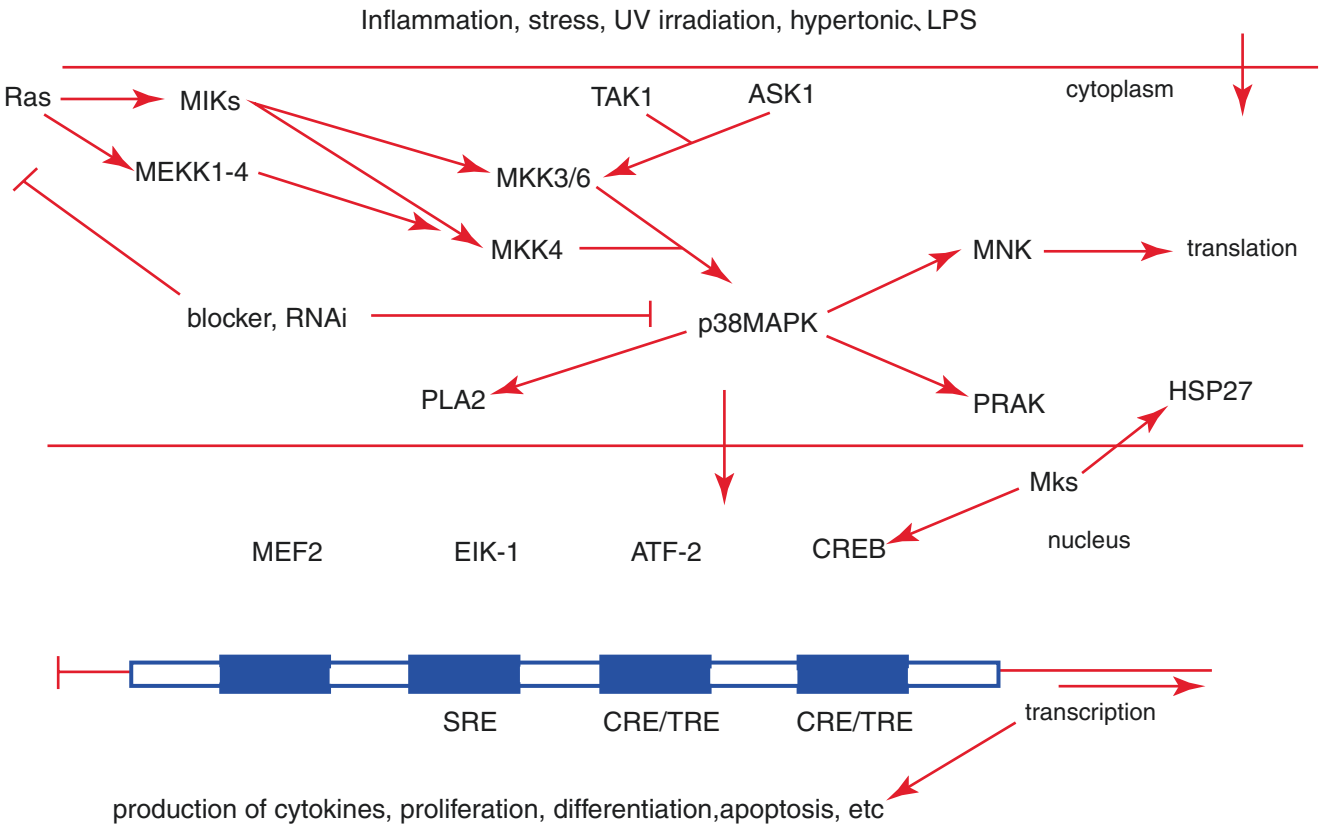

Fig. 3.7 Schematic diagram of the p38MAPK signaling pathway (Extracted from Zhang Qi, Bai Xiaodong, Xiaobing Fu. Progress in p38MAPK signaling pathway. Infection, Inflammation, Repair, 2005, 6(2): 121–123)

undergo apoptosis through caspase. Studies have shown that the number of neutrophils infiltrated from 0 to 12 h gradually increases, and the number of cells and the rate of positive cells reach the peak, suggesting that after skin damage, the body's defense system was started, and the number of apoptotic neutrophils was also gradually increasing. At 12 h, p38MAPK-mediated neutrophil apoptosis was the most, which may be related to the stimulation of inflammatory factors, leading to rapid apoptosis of neutrophils. The number of neutrophils decreased significantly in 1 to 3 days, the number of monocytes increased gradually, and a large number of fibroblasts proliferated, indicating that the tissue could eliminate too many neutrophils by phagocytosis of monocytes/macrophages to avoid tissue damage. The positive cell rate was relatively high in 1 ~ 5 days, the highest in the third day, indicating that this may be the peak of monocyte apoptosis. And studies have shown that the peak of caspase-3 expression is also on the third day after skin injury. Other studies have confirmed that both mononuclear macrophages and fibroblasts express Fas/FasL during skin injury healing, and some cells co-expressing Fas/FasL have apoptosis. The expression of p-p38MAPK was found during the apoptosis of macrophages, and it was found that transforming growth factor (TGF-β) and inducible NO synthase (iNOS) can activate p38MAPK through different pathways, and then initiate exogenous cell death pathway Fas/FasL and endogenous cell death pathways p53 and Bax, and finally induce macrophage apoptosis through caspase-3. In addition, studies have shown that the differentiation and chemotaxis of monocytes in serum is also mediated by the activation of p38MAPK. This indicates that p38MAPK can transmit different signals to regulate the function of monocytes. Under normal circumstances, 5 days after injury, monocytes decreased and fibroblasts increased gradually. In this stage, the number of cells was small and the tissue entered the reconstruction stage. After 5 ~ 14 days, the positive expression of fibroblasts increased first and then decreased gradually. Finally, the expression of positive cells and the content of p38MAPK approached normal skin. At this stage, the ERK pathway plays an important role in accelerating wound healing, and p38MAPK may mediate fibroblast apoptosis via Fas/FasL and may be based on dephosphorylation of the ERK pathway. In addition, in proliferative scar tissue, the protein expression of p38MAPK is higher than that of normal skin. This may be that after stimulating cells by inflammatory factors, p38MAPK can upregulate the expression of c-Jun gene, induce the synthesis and secretion of basic fibroblast

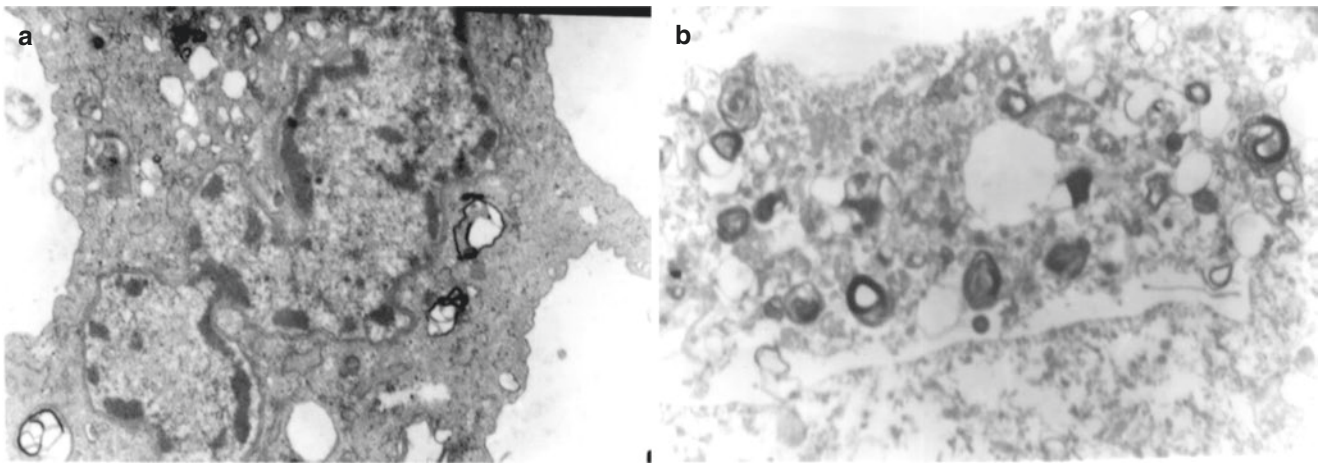

Fig. 3.8 (**a**) Nuclear chromosome collected and aggregated to form a crescent (transmitted electron microscopy) (**b**) Apoptotic bodies formed after thermal damage (transmitted electron microscopy)

growth factor (bFGF) and other factors, and promote the excessive proliferation and fibrosis of granulation tissue.

The team led by Xiaobing Fu first explored the activation of MAPK signaling pathway in apoptosis induced by thermal injury in vitro [56]. First, a scale model of fibroblasts in vitro was made. The primary human fibroblasts were cultured by subculture (selecting cells from 6 to 8 generations). When the density of inoculation reaches 10^5–10^6/mL, the DMEM medium with 10% serum concentration was changed to the DMEM with 5% serum concentration. The culture was continued for 24 h at 37 °C, 5% CO2, and saturated humidity. The cultured fibroblasts were placed in water baths at 43 and 45 °C for 10, 30, and 40 min, respectively. The apoptosis characteristics were most obvious after incubation in constant temperature water bath at 45 °C for 10 min. Under the inverted microscope, the cells of the normal control group were fusiform, with full body and smooth and bright membrane; after the thermal damage, some cells became round and appeared protuberance on the surface, that is, bubble phenomenon. DNA gel electrophoresis showed that the cells were incubated in water at 37 °C for 30 min (the normal control group) without any bands, and the heat-damaged group had DNA fragments, which showed typical ladder-like markers of apoptosis. Under the fluorescence microscope of Hoeschst33258, the living cell nucleus showed diffuse and uniform fluorescence, while the apoptotic cells, the nucleus, and cytoplasm showed dense particle fluorescence, especially 3–4 fluorescent fragments, which is considered to be typical apoptotic cells. Under transmission electron microscopy, the nuclear chromosomes of apoptotic cells were collected and aggregated to form a crescent. Some cells had pyknosis, increased electron density, irregular nuclear shape, and uneven nuclear membrane surface. Some cells underwent nuclear fragmentation, and multiple electron density-enhanced nuclear fragments appeared in the cytoplasm (Fig. 3.8). Some cells became smaller in size, the cytoplasm was concentrated, the organelles were well preserved, or slightly hyperplasia, the number of mitochondria was slightly increased and mildly swollen, the cytoplasmic vacuoles were increased, the cell membrane was preserved intact, and the surface microvilli and pseudopods were reduced or disappeared, and the bubble phenomenon of cell membrane can be seen. In the late stage of apoptosis, apoptotic bodies with intact organelles and nuclear fragments can be seen.

Using the above studies to make cell damage in vitro, it was observed that ERK1/2 changed after the simple injury, and the change of ERK1 was more significant. At 30 min, ERK1/2 expression reached the peak, and after 180 min, expression was almost complete. With bFGF stimulation, the expression level of each phase was enhanced, which was significantly higher than that of the control group. At 30 min, ERK1/2 expression was the strongest, peaked, and then began to diminish. The ERK pathway was specifically blocked in advance, and ERK expression was decreased after adding PD98059. Phosphorylated ERK expression: The phosphorylated ERK at each phase was lower than the total expression. When bFGF stimulator was added, the peak value of ERK at 30 min was significantly higher than that in the simple injury group, especially the expression of ERK-2. After blocking with the blocker PD98059, the phosphorylated ERK1/2 protein was not expressed at 0 ~ 180 min.

After induction of scald in vitro, the expression of proto-oncogene c-Fos in cultured fibroblasts increased gradually (0–30 min), decreased after 60 min, and weakened after 180 min. When adding exogenous growth factor bFGF, its expression increased and lasted for 180 min. Use ERK-specific blocker ahead of time PD98059, c-Fos expression was not significantly reduced compared with the simple scald-induced group, and the duration of sustained expression was prolonged. However, c-Fos expression was signifi-

cantly reduced after blocking both ERK and p38MAPK pathways with PD98059 and SB203580 antagonists.

In vivo, the team led by Academician Xiaobing Fu did the following study [57]. Twenty-six male Wistar rats weighing 160 ~ 190 g were purchased from the Institute of Laboratory Animals Science, CAMS & PUMC (license number: SCXK11000006). The diabetic rat model was prepared by the Institute of Materia Medica Chinese Academy of Medical Science. One week before the experiment, the rats were fed in the laboratory, and on the day of experiment, they fasted. Under the condition of ketamine and SU-XIN-MING compound anesthesia, the hair on the back was cleaned, with an area of 9.0 cm × 8.0 cm. After disinfection, 4 round wounds were cut on both sides of the back spine with a diameter of 1.8 cm and an area of 2.54 cm^2 by a special wound causing device (perforator). After hemostasis, 52 wounds were selected (the rest of the wounds were reserved for other purposes). They were randomly divided into two groups:

(a) Control group (group A), dressing with sterile gauze, feeding in single cage, and changing gauze once a day until the wound healed.
(b) rhPDGF treatment group (group B): rhPDGF is REGRANEX produced by a company in the United States, and the dosage of wound surface is 7.0 μg/cm^2. The rhPDGF was evenly applied to the corresponding wound surface with a syringe according to the experimental design dose, wrapped with sterile gauze, fed in a single cage, and changed dressing once a day until the wound healed. The blood glucose level of the tail vein was measured by a portable blood glucose meter on the 3rd, 7th, and 14th day after the injury. The granulation formation, collagen deposition, re-epithelialization rate, and inflammatory cell infiltration were observed on the 3rd, 7th, and 14th day after injury, the expression of extracellular signal-regulated kinase 1/2 (ERK1/2) was detected by immunofluorescence technique, and proto-oncogene c-Fos, proliferation cell nuclear antigen (PCNA), focal adhesion kinase (FAK) changes were detected by immunohistochemistry technique. Histological examination showed that there were a large amount of inflammatory cells infiltrated in the wound treated with rhPDGF, the number of capillary germ and fibroblasts were significantly higher than those in the control group, collagen deposition was obvious, granulation tissue grew actively, and wound contraction was significant (Fig. 3.9). Immunological studies showed that the expression of ERK1/2 and c-Fos was significantly increased in the rhPDGF-treated group 3 days after injury, ERK1/2 and c-Fos were further enhanced at 7th day, and began to weaken after 14 days. The expression of PCNA and FAK in the repaired cells of rhPDGF-treated group was significantly stronger than that of the control group. It can be seen that the early use of rhPDGF can induce the expression of ERK1/2 in repair cells (including fibroblasts, vascular endothelial cells, and keratinocytes), thereby increasing the transcription and expression of proto-oncogene c-Fos. According to the rule of experimental wound healing and the role of influencing factors, the researchers speculated that ERKs control the expression of downstream genes by regulating the activity of AP-1. AP-1, as a transcription factor for FAK and PCNA expression, affects the biological activities of various repair cells, including fibroblasts, microvascular endothelial cells, and keratinocytes, especially the proliferation, migration, and differentiation of

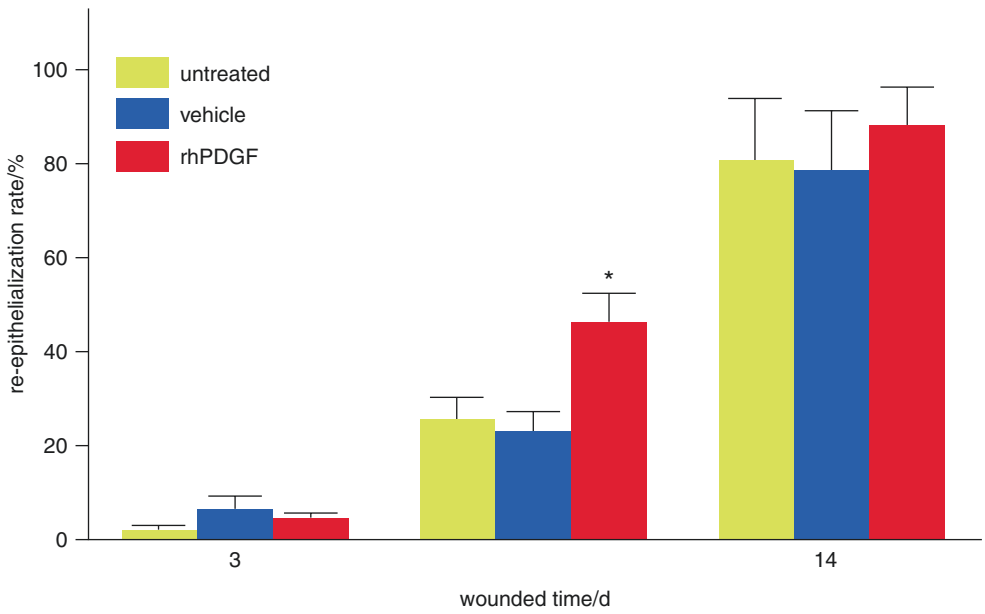

Fig. 3.9 Effect of PDGF on the rate of re-epithelialization in diabetic rats

repair cells, and finally accelerates the collagen deposition, blood vessel formation, and re-epithelialization in granulation tissue, and promotes wound healing process. PDGF is a strong mitogenic agent, which induces the migration of wound vascular smooth muscle cells, the proliferation and migration of endothelial cells, and the formation of vascular lumen, and is beneficial to the oxygen supply of tissues; it promotes the proliferation of fibroblasts and increases the composition of extracellular matrix to facilitate tissue contraction; it promotes proliferation and migration of keratinocytes, and contributes to the re-epithelialization of wounds. In conclusion, rhPDGF-induced chemotaxis and migration of repair cells is an important component of the wound healing response. The ERK signal transduction pathway is the main pathway for PDGF to stimulate angiogenesis in granulation tissue. Fos protein is a key product involved in signal cascade. Further elucidation of the regulation mode and network characteristics of PDGF in the MAPKs signaling pathway will play an important role in understanding the mechanism of wound healing.

HE staining of wound tissue sections showed that the amount of inflammatory cells infiltrated in the control group of diabetic rats was much less than that in the rhPDGF-treated group on the 3rd day after injury. After treatment with rhPDGF, the inflammatory infiltration zone of wound was obvious, and a large number of inflammatory cells (macrophages, monocytes, and neutrophils) appeared in the injured area. The wounds in different groups showed different degrees of granulation tissue proliferation on the 7th day after injury. The collagen deposition in the wounds treated by rhPDGF was more, and the number of capillary germ and fibroblasts increased significantly (Table 3.1). On the 14th day after injury, the wound contraction was significant after rhPDGF treatment, and re-epithelialization was almost completed, while other groups were still not completely healed. Further observation of their changes in ERK1/2 expression revealed that phosphorylated ERK1/2 positive markers appeared in repair cells (fibroblasts, microvascular endothelial cells in the wound, and keratinocytes in the basal layer of wound edge epithelium) on the 3rd day after injury. Expression increased on the 7th day and remained high on the 14th day. The expression of ERK1/2 was enhanced after treatment with rhPDGF in group B. The number of ERK1/2 positive cells was significantly increased compared with the diabetic control group on the 3rd day after injury. There were still a large number of ERK1/2 positive cells in 7–14 days after injury. The study on the expression of proto-oncogene c-Fos revealed that the expression of proto-oncogene c-Fos in various repair cells of rats increased gradually after injury, and the number of positive cells reached the peak on the 7th day after injury; there are still a large number of positive cells on the 14th day. After rhPDGF was applied to the wound, the number of positive cells at each time point increased further, exceeding the diabetic control group. The general condition and pathological observation of diabetic rat skin defect wound treated by external rhPDGF shows the healing mechanism of the study.

Table 3.1 Comparison of wound volume at different time points after treatment with different methods

Group	3 d	7 d
Diabetic rats control	0.21 ± 0.06 (n = 29)	0.07 ± 0.05 (n = 25)
PDGF	0.18 ± 0.04 (n = 12)*	0.03 ± 0.03 (n = 11)*

Compared with the diabetic control group: *$P < 0.05$; n is the number of wounds

Subsequently, the team led by Xiaobing Fu further explored the expression of phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), phosphorylated stress-activated protein kinase (p-SAPK), and phosphorylated p38MAPK (p-p38MAPK) during the formation and maturation of hypertrophic scars after wound healing [58]. 16 cases of hypertrophic scars were collected from hospitalized patients, including nine males and seven females, aged from 16 to 47 years old. The materials included five cases of the back, one case of the buttock, three cases of the maxillofacial and upper limbs, respectively, and two cases of the chest and inner thigh, respectively. The hypertrophic scar specimens were divided into two groups, 8 in each group:

(a) Hypertrophic scar in active growth period with formation time less than 1 year (including 1 year).
(b) Mature hypertrophic scar with formation time more than 1 year. Eight cases of normal skin were taken from normal skin of patients with hypertrophic scar resection. It was found that p-ERK1/2, p-SAPK, and p-p38MAPK were positively expressed in normal skin, among them p-ERK1/2 and p-SAPK were less expressed, and the protein particles were mainly distributed in the cytoplasm and nucleus of keratinocytes in the basal layer of the epidermis. The expression of p38MAPK protein is strong, and the positive staining signal is mainly located in keratinocytes, vascular endothelial cells, hair follicles and sweat gland epithelial cells, and some fibroblasts. In hypertrophic scars, the expression of all three proteins was enhanced, and the protein particles of p-ERK1/2 and p-SAPK were mainly localized in epidermal keratinocytes and a small number of fibroblasts; p-p38MAPK was strongly expressed, mainly in epidermal cells and fibroblasts. Studies have shown that in hypertrophic scars, the expression of p-ERK1/2 protein is significantly higher than that in normal skin, and positive signals were mainly distributed in epidermal basal cells, which were mainly epidermal stem cells and transiently expanded cells. This suggests that in hypertrophic scars,

extracellular signals can activate the signal transduction pathway mediated by ERK1/2 phosphorylation, causing rapid proliferation of epidermal stem cells and transiently expanded cells, and at the same time a large number of terminally differentiated cells are formed. As these cells synthesize and secrete extracellular matrix such as collagen, hypertrophic scars continue to expand and mature. In the active hypertrophic scar, the p-SAPK protein content was not significantly different from normal skin, but in the mature hypertrophic scar, the p-SAPK positive cell rate was significantly higher than that of normal skin, and mainly located in the epidermal basal cells and parts of fibroblasts. This suggests that as hypertrophic scars mature, extracellular stress signals (such as hypoxia and nutrient deficiency) increase, activating the SAPK signaling pathway, thereby slowing cell division, partial fibroblast apoptosis, and ultimately leading to the balance of proliferation and apoptosis of scar-forming cells, and hypertrophic scars are in a relatively stable state. In the active hypertrophic scar, the expression of p-p38MAPK protein was significantly higher than that in normal skin, and its mechanism may be related to the strong inflammatory reaction and the high content of proinflammatory cytokines in the early stage of hypertrophic scar formation. The specific binding of extracellular signals and receptors on the cell membrane can activate p38MAPK signaling pathway, upregulate c-Jun gene expression, induce the synthesis and secretion of factors such as bFGF, promote hyperplasia and fibrosis of granulation tissue, and form hypertrophic scars. In mature hypertrophic scars, the expression of p-p38MAPK protein is decreased compared with that in active scars, but it is still higher than normal skin. The mechanism of this change may be related to the decrease of inflammatory cells and inflammatory response in mature hypertrophic scars. The p38MAPK signaling pathway may play an important regulatory role in the maintenance of mature hypertrophic scars.

3.4.2 Phosphatidylinositol-3-Kinase Signaling Pathway

3.4.2.1 Structural Characteristics and Activation Regulation of PI3K

Phosphatidylinositol-3-kinase (PI3K) is a heterodimer composed of a catalytic subunit p10 and a regulatory subunit p85. It has lipid kinase activity and protein kinase activity, and the p85 regulatory subunit is a phospholipid protein substrate of many receptor tyrosine kinases. According to the PI3K structure, it can be divided into three types (type I, type II, and type III): PI, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-diphosphate (PIP2) are used as substrates for type I PI3K; PI and PIP are used as substrates for type II PI3K, including PIK3C-α, PIK3C-β, and liver-specifically expressed isoform PIK3C-γ; type III PI3K consists of catalytic subunit Vps34 and regulatory subunit p150, and PI is the substrate, mainly involved in the regulation of cell growth and survival. Currently, the most widely studied is type I PI3K, which is activated by cell surface receptors. Type I PI3K is divided into two subtypes, I A and I B, which transmit signals from tyrosine protein kinase-coupled receptor and G-protein-coupled receptor, respectively. I A type PI3K has dual activities of phosphatidylinositol kinase and serine-threonine protein kinase. It is a heterodimer composed of catalytic subunit p110 and regulatory subunit p85. The catalytic subunits include p110α, p110β, and p110δ, which are encoded by PIK3CA, PIK3CB, and PIK3CD genes, respectively. Studies have shown that the PI3K/Akt pathway is independent of PKC, PKA, and MAPK, and is involved in the regulation of various tissue activities including angiogenesis, and plays a crucial role in cell proliferation, differentiation, and apoptosis.

The activation of PI3K is largely involved in substrates close to the inside of its plasma membrane. A variety of growth factors and signal transduction complexes, including fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and insulin, all initiate the activation of PI3K. These factors activate the receptor tyrosine kinase (RTK), which causes autophosphorylation. The phosphorylated residue at the receptor provides a docking site for the heterodimerized PI3Kp85 subunit. In some cases, receptor phosphorylation mediates the recruitment of an adaptor protein. When integrin is activated, focal adhesion kinase (FAK) acts as an adaptor protein, anchoring PI3K through its p85. However, in each of the above cases, the SH2 and SH3 domains of the p85 subunit bind to the adaptor protein at a phosphorylation site. Phosphorylation of various PI intermediates is initiated after PI3K recruits activated receptors.

3.4.2.2 The Relationship Between PI3K/Akt and Various Growth Factors Related to Wound Healing

PDGF is mainly derived from platelet alpha particles, which can cause proliferation and migration of various cells such as fibroblasts, smooth muscle cells, and monocytes. It is an important growth factor and plays an important role in wound healing, especially angiogenesis. Studies have confirmed that PDGF promotes the proliferation of vascular smooth muscle cells by activating the Akt/FoxO1/3a signaling pathway.

FGF-2 antagonizes apoptosis induced by oxidative stress by activating the PI3K/Akt signaling pathway. In mouse ESCs, FGF activates ERK signaling pathway to promote

ESC differentiation, and inhibits ERK signaling pathway to promote self-renewal of mouse ESCs. Enzyme-linked immunosorbent assay (ELISA) analysis showed that bFGF can activate the Wnt pathway. RNA sequence analysis of β-catenin knockdown fibroblasts showed that β-catenin was positively regulated by bFGF and FGF21. In addition, FGF21 activates Akt and JNK, and its effect of accelerating fibroblast migration is similar to bFGF. Furthermore, ELISA analysis indicated that bFGF and FGF21 autocrine were regulated by stimulation of the Wnt pathway. In conclusion, in skin fibroblasts, β-catenin is involved in the regulation of bFGF (FGF21) and Wnt signaling in dermal fibroblasts.

EGF promotes proliferation of epithelial cells, fibroblasts, glial cells, and smooth muscle cells. EGF induces the phosphorylation of EGFR on the cell surface and activates Akt in the PI3K/Akt pathway of inositol triphosphate kinase, which is closely related to cell proliferation. Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes that are required for cell-mediated contraction in cell migration and wound healing. It has been reported that matrix contraction and cell migration can be reduced by inhibiting the activity of MMPs. After activation, gelatinase A (MMP-2) secreted by connective tissue cells can degrade one or more collagens and proteins. It has the property of degrading denatured collagen gelatin and has certain effects on fibronectin and elastin. Studies have shown that EGF induces overexpression of MMP-2 in cultured human lens epithelial cells (HLECs) in a time-dependent manner and completes cell migration, which can be blocked by EGFR and Akt inhibitors. These results indicate that PI3K/Akt channel is a necessary condition for EGF to stimulate MMP-2 expression.

Transforming growth factor (TGF) is divided into TGF-α and TGF-β. The former amino acid sequence is 33–44% homologous to EGF, which can bind to EGF receptor and exert the same or similar effects. The latter effect on fibroblasts and smooth muscle cells proliferation varies according to its concentration: low concentration induces PDGF synthesis and secretion, which is an indirect mitogen; high concentration inhibits PDGF receptor expression and inhibits its growth. Many studies have shown that TGF-β and PI3K/ Akt signaling pathways interact at multiple levels and multiple steps to achieve fine-grained regulation of cells in specific environments.

Vascular endothelial growth factor (VEGF) is a highly selective and potent mitogen for endothelial cells. Previous studies have suggested that it can specifically act on vascular endothelial cells, induce the expression of matrix metalloproteinases and collagenases, promote the degradation of extracellular matrix, and facilitate the migration of endothelial cells, thereby inducing the migration of endothelial cells and preparing for the generation of new blood vessels. Hypoxia-inducible factor-1 (HIF-1) acts as a mediator of transcription activation during hypoxia. Growth factors, cytokines, etc. stimulate the synthesis of HIF-1α by activation of PI3K or MAPK. HIF-1 promotes the generation of new blood vessels by binding to the VEGF promoter hypoxia-responsive element (HRE) to regulate the expression of VEGF and the transcription of its receptor. Overexpression of PI3K or Akt increased VEGF mRNA expression, while LY294002 inhibited VEGF mRNA expression, but overexpression of PI3K or Akt restored inhibition. This suggests that PI3K may induce angiogenesis by regulating the expression of HIF-1 or VEGF.

Insulin-like growth factor 1 (IGF-1) is a single-chain polypeptide produced and secreted mainly by hepatocytes. It induces the expression of growth factors such as basic fibroblast growth factor, promotes the synthesis and secretion of extracellular matrix such as fibronectin, polyglucosamine, and collagen, and affects the remodeling of tissue after wound repair. It can stimulate the mitosis of the mother cell by binding to the specific IGF-1 receptor. Studies have suggested that plasma IGF-1 levels in diabetic patients are significantly lower than in normal controls, while IGF-1 levels in patients with high risk of diabetic foot ulcers are lower than those in patients without risk and low risk, that is, with the increase of the risk of diabetic foot ulcer, the level of plasma IGF-1 gradually decreases, indicating that the change of plasma IGF-1 level is closely related to the occurrence and development of diabetic foot ulcer. Ulcer healing in diabetic patients is slower than normal, indicating that IGF-1 plays an important role in the healing process of ulcers. Many studies have demonstrated that IGF-1-mediated multiple cellular functions may be primarily implemented through the PI3K/Akt signaling pathway. The binding of IGF-1 to the IGF-1 receptor leads to the activation of two signaling pathways, namely mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt), while activating the PI3K/Akt signaling pathway can affect cell apoptosis. The mechanism of PI3K/Akt signaling pathway interfering with apoptosis mainly includes the following aspects:

(a) Direct regulation. Activated Akt can phosphorylate the Ser136 site of Bad, effectively block Bad-induced apoptosis, inactivate the caspase-9 Ser196 site, and inhibit its pro-apoptotic effect.
(b) Play a role in cell survival regulation by directly or indirectly affecting the transcription factor family (Forkhead, NF-κB, p53, etc.).
(c) Influence cell proliferation by regulating cell cycle.
(d) Prevent mitochondria from releasing apoptotic factors. The protective effect of IGF-1 on cardiomyocytes is mainly through activating PI3K/Akt pathway. The PI3K/Akt pathway is a classical pathway that mediates survival.

3.4.3 Wnt Signaling Pathway and Tissue Repair

In 1973, Sharma et al. first discovered the wingless gene in the study of drosophila embryo development. In 1982, Nusser et al. discovered a protein that can transmit proliferation and differentiation signals between cells when studying mouse breast tumors. At that time, it was called Intl. Later, it was found that the wingless gene of drosophila is a Wnt-like gene, which is later named Wnt1 gene.

At first, Wnt gene family was composed of wingless gene (Wg) products and the secreted glycoprotein Wnt1 gene. Wnt glycoprotein regulates homeostasis and development by binding to the Frizzled-low density lipoprotein receptor-associated protein complex. The Wnt signaling pathway is evolutionarily conserved and is a highly complex signaling pathway. It is also the key for cell development, differentiation, and maintenance of homeostasis. It is an important signaling pathway for controlling embryonic development and morphogenesis of tissues and organs. A total of 19 Wnt genes have been discovered, which are expressed in a variety of tissue cells. They act by activating the membrane receptor in an autocrine or paracrine manner. The Wnt protein encoded by the Wnt gene, its receptor, and regulatory proteins, together form a complex signaling pathway called the Wnt signal transduction pathway. The Wnt signal transduction pathway has the following two pathways:

(a) Classical Wnt-β-catenin-LEF/TCF pathway: After activation, this pathway will recruit β-catenin in cells, activate and transfer it into the nucleus, and activate the transcription of specific genes together with transcription factor LEF/TCF. β-catenin was first discovered by German cell biologist Walt Birchmeier in 1980 as a component of the cell adhesion connection. It is a member of the cytoskeletal protein family. The β-catenin protein contains three important functional regions: the N-terminal domain, the intermediate linking arm repeat region, and the C-terminal domain. The amino terminus contains about 150 amino acids. It is the phosphorylation site of β-catenin by glycogen synthase kinase 3β (GSK3β) and also a binding site to α-catenin. Among them, several serine/threonine residues can be phosphorylated by GSK3β and CK1α, and phosphorylated β-catenin can be recognized and ubiquitinated by β-transducin repeat-containing proteins, and finally degraded in the proteasome. It is one of the important mechanisms regulating the stability of β-catenin. The intermediate repeat region is a sequence with the same structure, which is the most conserved region and the main binding site with ligand, including β-catenin and cadherin, Axin, lymphoid enhancer factor (LEF), etc., thereby activating the T-cell factor (TCF) family transcription factor binding site at the carboxy terminus of the classical Wnt signaling pathway, and activating downstream target genes to play the role of transcription after binding to a transcriptional activator.

(b) Nonclassical Wnt signaling pathway: With the deepening of Wnt signaling pathways, more and more Wnt nonclassical signaling pathways have been discovered, including Wnt-PCP signaling pathway, Wnt-cGMP signaling pathway, Wnt/Ca^{2+} signaling pathway, Wnt-PAP signaling pathway, etc. The study of PCP pathway and Ca^{2+} pathway is clear in these pathways.

Under normal circumstances, Wnt gene of adult is in a relatively static state. After skin injury, TGF-β increases β-catenin expression in the wound. TGF-β activates β-catenin-mediated transcription of human epithelial fibroblasts via the Smad3 and p38 MAPK pathways, and TGF-β also induces upregulation of Wnt/β-catenin signaling pathway in hypertrophic scars and keloids. β-catenin is continuously increased in the nucleus of dermal fibroblasts, which is beneficial to the proliferation and migration of fibroblasts, and at the same time, it can feedback to activate TGF-β signaling pathway. The enhanced Wnt/β-catenin signaling pathway plays a negative feedback role in the transformation of normal skin from fibroblasts to myofibroblasts induced by TGF-β1, which is the key to wound healing.

The interaction between endothelial cells is an important factor affecting the vascular proliferation and vascular function of wounds. Studies have shown that VEGF and placental growth factor promote vascular proliferation through the receptor VEGFR-1, and the two have a synergistic effect. Blocking Wnt/Ca^{2+} signaling pathway by siRNA or Wnt5a antagonist can inhibit endothelial cell proliferation and migration, and the addition of VEGF can reverse this phenomenon. It is suggested that Wnt5a-mediated nonclassical Wnt pathway (Wnt/Ca^{2+}) plays a positive role in endothelial cell proliferation and migration.

Wnt signaling pathway is activated after skin injury and is involved in every process of wound healing from inflammation control to apoptosis. The relationship between Wnt signaling pathway and wound healing is illustrated in the following aspects.

3.4.3.1 Inflammatory Cells

Inflammation-related studies in wound healing have found that interferon-γ (IFN-γ) and lipopolysaccharide (LPS), as two potent inflammatory stimulators, can significantly upregulate Wnt5a. Studies on the relationship between macrophages and Wnt signaling pathways suggest that direct evi-

dences of macrophage-stimulating Wnt signaling pathways include the following: macrophages can stimulate the remodeling of vitreous vessels, and Wnt7b secreted by macrophages is a very important protein involved in the entire process. Moreover, Wnt5a can promote macrophages to produce cytokines with proangiogenic effects, such as IL-6, IL-8, and the like.

3.4.3.2 Fibroblasts

Zheng Fang et al. found that the proliferation and secretion of fibroblasts is beneficial to the repair of wounds, and Wnt signaling is involved in the proliferation of fibroblasts during wound healing, especially through the Wnt/β-catenin signaling pathway. Carre et al. found that the classical Wnt signaling pathway is significantly increased in the wound surface of newborn mice. After Wnt signaling pathway is activated, it could promote the proliferation of mouse fibroblasts after birth and increase the expression of HAS1 and Hyal2 genes in postnatal mouse fibroblasts, thereby significantly increasing the expression of type I collagen. β-catenin is involved in the proliferative phase of wound repair in rats and humans. It is believed that β-catenin plays a regulatory role in the movement of fibroblasts, while abnormal β-catenin can cause excessive fibrous tissue and scar formation. The phosphorylation of β-catenin, its accumulation in the cytoplasm, the transfer into the nucleus, and the regulation of target gene transcription can cause fibroblast proliferation, migration, and collagen deposition. The changes of β-catenin level in fibroblasts can regulate wound contraction, tensile strength, and TGF-β.

3.4.3.3 Angiogenesis

New blood vessels provide adequate blood oxygen supply and nutrition for wound healing, which is conductive to granulation growth and wound healing. Therefore, the effect of Wnt signaling pathway on angiogenesis deserves attention. Studies have shown that the Wnt signaling pathway is involved in the regulation of vascular endothelial cell proliferation and plays an important role in maintaining the function of endothelial cells and endothelial progenitor cells. Wnt1, Wnt3a, and Wnt5a can control the proliferation, migration, and differentiation of endothelial cells. Endothelial cell proliferation and migration are inhibited by Wnt antagonist or siRNA blocking Wnt signaling pathway, which may be reversed by the addition of vascular endothelial growth factor (VEGF). It has been reported that Wnt can induce the expression of matrix metalloproteinases, promote the degradation of extracellular matrix, and facilitate the recognition and establishment of connections of sophisticated cells. In vitro experiments with vascular smooth muscle cells have shown that it can promote the degradation of β-catenin/cadherin complex at the adhesion junction and affect the migration of endothelial cells into new blood vessels.

3.4.3.4 Epidermal Regeneration and Hair Follicle Regeneration

Epidermal regeneration and hair follicle regeneration play a very important role in the repair of wounds, and Wnt is involved in many processes of skin development, from dermogenesis to skin appendage formation. Fathke et al. [59] pointed out Wnt classical signaling pathway can promote the proliferation of hair follicle stem cells and promote hair follicle stem cells into the cell cycle and differentiate into corresponding cells according to different regulatory signals. Hair follicle stem cells have multidirectional differentiation potential and can differentiate into epidermis, sebaceous glands, and hair follicles. Wnt/β-catenin is essential for the development of hair follicles during embryogenesis and contributes to the differentiation of hair follicles after birth. Wnt signaling plays an important role in hair follicle regeneration after injury. For example, Langton et al. found that rats with tail skin damage and lack of hair follicles still had re-epithelialization, but this process was delayed. Inhibition of Wnt signaling in skin lesions will prevent the formation of skin appendages, including hair and sweat glands, which can cause permanent scar formation.

3.4.3.5 Stem Cells

The Wnt signaling pathway affects stem cell proliferation and self-renewal, which is closely related to wound healing. These stem cells are important for tissue repair and regeneration. The Wnt signaling pathway regulates the recruitment and differentiation of stem cells during wound repair. Interstitial progenitor cells also contribute to the repair of wounds. Mesenchymal progenitors at different stages of differentiation are regulated by Wnt signaling pathway, and the differentiation of these cells can be affected by the change of signaling pathway activity, thus affecting the differentiation of these cells. The dermal papilla is the expression area of many genes related to hair growth. Many experiments have demonstrated that Wnt proteins and growth factors can stimulate the dermal papilla by promoting stem cell migration. Small doses of Wnt can induce differentiation of mesenchymal stem cells, while large doses of Wnt inhibit differentiation of mesenchymal stem cells. The terminally differentiated cells that do not migrate accumulate around the wound to hinder epithelialization. Blocking of the Wnt signaling pathway reduces the recruitment of stem/progenitor cells in the damaged area and affects the proliferation of the healing process. Whyte speculated that it might be due to changes in oxygen concentration, and speculated that HIF-1 might be the target of injury and Wnt signaling activation.

3.4.3.6 The Role of Wnt/β-Catenin Signal Transduction Pathway in Pathological Scar Formation

The Wnt/β-catenin signal transduction pathway plays an important role in the healing process of skin wounds and is closely related to human fibrotic diseases. Emily et al. found that the expression of β-catenin was increased in keloid tissue. It is speculated that dermal hyperplasia is caused by the interaction of β-catenin with dermal cells, which is highly expressed in the epidermis. It is confirmed that β-catenin is involved in various stages of wound healing at multiple levels. Li et al. found that Wnt/β-catenin signaling pathway is closely related to cell carcinogenesis and tumor invasion. Pathological scars can be identified as a benign skin fibrotic tumor with tumor biological properties. Igota et al. found that the levels of Wnt5a mRNA β-catenin and protein in keloid fibroblasts were higher than those in normal skin fibroblasts. Domestic scholars have confirmed that β-catenin, which is highly expressed in pathological scars, activates the Wnt/β-catenin signal transduction pathway and promotes the formation of scars. Yasuniwa et al. found that Wnt10A has the effect of promoting tumor angiogenesis. Later, some scholars found that keratinocytes of keloids may induce the high expression of R-spondin2 in fibroblasts, and R-spondin2 cooperates with Wnt3a through the classical Wnt/β-catenin signal transduction pathway. O-gawa et al. believe that skin tension is an important factor in triggering scar formation. Reducing skin tension around wounds or scars is an effective measure to prevent and treat pathological scars. The formation and development of pathological scars may be related to local skin tension. Stretched cells migrate faster and farther than unstretched cells, with no proliferation and reduced apoptosis, and at the same time, the synthesis of collagen is unchanged and the degradation is increased. These biological effects are mediated by integrin and Wnt signaling pathway.

The team led by Xiaobing Fu explored the effects of Wnt/β-catenin signaling pathway activation on human epidermal cell phenotype changes [60]. They used differential adherence method to separate human mature epidermal cells and divided the cells into control group and induction group. The induction group was further divided into lithium chloride (LiCl, 20 mmol/L) induction group and GSK-3β inhibitor (15 μmol/L) induction group. The morphological changes of the cells were observed after 6 days of induction, the expression level of β-catenin in epidermal cells was detected by Western blot, and the expression of surface markers such as CK10, CK14, CK19, and β1 integrin were detected by immunohistochemistry. The results showed that the expression of β-catenin in human epidermal cells induced by LiCl (2.15 ± 0.54) and GSK-3β inhibitor (2.58 ± 0.65) was 2 and 2.5 times higher than that in the control group (0.71 ± 0.23), respectively ($P < 0.01$). Moreover, after induction, the cell volume became smaller and rounder and the ratio of nuclei and cytoplasm became larger. The results of immunohistochemistry showed that CK10 was highly expressed in epidermal cells before induction, and CK14, CK19, and β1 integrin expression were negative in some cells. After induction, there were no CK10 positive cells, some CK14 positive cells, and the expression of CK19 and β1 integrin was strongly positive. This indicated that Wnt/β-catenin pathway activation can cause dedifferentiation of human mature epidermal cells, and Wnt/β-catenin pathway may be the key link regulating human epidermal cell dedifferentiation.

If the molecular mechanism of regulating epidermal stem cell proliferation and differentiation is further clarified, it is possible to precisely regulate the limited proliferation and directed differentiation of epidermal stem cells by changing different gene signals in the Wnt pathway, thus providing new ways and means for the research of skin regeneration medicine and tissue engineering.

3.4.4 TGF-β/Smads Signal Transduction Pathway

3.4.4.1 TGF-β Structure, Secretion, and Activation

To date, at least six subtypes of TGF-βs have been found, namely TGF-β 1 ~ 6, and three subtypes have been found in mammals, namely TGF-β1, TGF-β2, and TGF-β3. These three factors have similar biological activities, inhibit proliferation of most types of cells, induce apoptosis of epithelial cells, but stimulate the proliferation of mesenchymal cells to produce extracellular matrix, and induce various tissue fibrosis reactions in vivo. TGF-β must be activated after secretion to exert its effect, that is, the TGF precursor molecule LTGF must be activated into mature TGF-β (TGF-β1, TGF-β2, TGF-β3) forms to connect with the receptor, and then activate signal transduction pathway. Unlike most other hormones, mature TGF-β remains covalent bond to its propeptide after secretion, and this complex form of mature TGF-β cannot be recognized by signal receptors, so it is called TGF-β propeptide. TGF-β signaling molecules conduct signal through transmembrane receptor complexes, which are widely present on a variety of cell surfaces. Their cell effects include extracellular matrix deposition, cell growth inhibition, and apoptosis. Extracellular matrix deposition involves stimulation of matrix proteins and inhibition of proteases.

The Smad protein family is a signal mediator molecule that transmits signals produced by the binding of TGF-β to its receptor from cytoplasm to nucleus. Numerous different Smad proteins have been found in mammals. These proteins can be divided into three different subfamilies: receptor-regulated Smads (R-Smad), common-partner Smads (C-Smad), and inhibitory Smads (I-Smad). All Smads

proteins consist of three parts: the highly conserved MH1 and MH2 domains at the amino and carboxy termini, and the proline-rich spacer linker (L). The MH1 region is a highly conserved amino terminus. In the ground state, the MH1 region of Smad2, Smad3, and Smad4 automatically inhibits the MH2 region and maintains it in an inactive state.

In the basic process of Smad-mediated TGF-β signaling transduction, TGF-β signaling is delivered to the nucleus in a non-amplified stoichiometric manner, requiring two single transmembrane serine/threonine receptors and Smad proteins. The dimeric ligand of the TGF-β superfamily has a high affinity for type I.

and type II receptors on the surface of the cell membrane to form a heterotetramer. In this complex, type II receptor phosphorylates autonomously, and it can phosphorylate the GS domain of type I receptor to activate it. Activated TGF-βR I phosphorylates two serine residues in the R-Smad SSxS region and combines with Smad 4 to form a heterotrimer or heterotetramer into the nucleus, and synergizes with a number of coactivators and coinhibitors to regulate transcription of the target gene.

3.4.4.2 The Role of TGF-β/Smads Signal Transduction in Wound Healing

As a multifunctional cytokine, TGF-β is closely related to wound healing. The team led by Academician Xiaobing Fu found that the biological functions of the three isoforms of TGF-β in the body were different [61]. Compared with TGF-β2 and TGF-β3, TGF-β1 is most closely related to wound healing. At the same time, the team found that TGF-β1 protein content in the central tissue of the ulcer decreased compared with normal skin and ulcer margins, and positive signals were only found in epidermal basal cells and macrophages. The contents of TGF-β2 and TGF-β3 protein were significantly increased, and TGF-β2 was expressed in almost all cells, especially in hair follicle cells; while TGF-β3 was rarely expressed in hair follicle cells, and it was obvious in epidermis, sweat glands, and vascular endothelial cells. Therefore, the reason of delayed wound repair may be the decrease of TGF-β1 content in the ulcer, and the increased expression of TGF-β2 and TGF-β3 protein, causing slow growth of granulation, insufficient blood supply, imbalance of matrix fibrin metabolism, etc., eventually leading to the occurrence of ulcer, but the specific mechanism still needs further study. At the ulcer wound, the content and distribution of receptor I protein were not abnormal compared with normal tissues. It can be seen that the occurrence of ulcer may be independent of the expression and distribution of TGF-βR protein.

TGF-β can promote the synthesis of extracellular matrix (ECM), which mainly contains collagen types 1 to 4. In the process of TGF-β promoting ECM synthesis, Smad regulates this process by interacting with COL1A1, 3A1, 5A2, 6A1, 6A3, and TIMP-1 genes. In addition, non-Smad-dependent pathways such as the JNK pathway have also been discovered. At the same time, it was found that Smad3 can promote type I collagen synthesis in human skin fibroblasts. From the above studies, it is concluded that Smad3 and its family are involved in collagen synthesis. But does collagen synthesis involved in Smad3 and its family play a role in wound healing? If collagen synthesis is inhibited by inhibiting Smad3 and its family, the effect of this inhibition on wound healing remains to be demonstrated. In conclusion, this signaling pathway plays an extremely important role in tissue repair and regeneration regulation.

3.4.5 Slit-Robo Signal Pathway

Slit is a secreted extracellular matrix protein discovered in 1984 by Nusslein-Volhard et al. in drosophila. It is encoded by gene 4p15.2 and has a molecular weight of about 200 ku (2.0 × 105). The Robo family is the receptor family of Slit, which is a single-channel transmembrane receptor. There are four members of the family in mammals, Robo1/Dutt1, Robo2, Robo3/Rig-1, and Robo4/Magic Roundabout. Slit binds to the Ig1-2 region of Robo via the N-terminal D2 region (second leucine repeat region). HSPGs is a co-receptor of Slit and Robo, which can combine with Slit2 and Robo to form a ternary compound (Slit- Robo-heparin) to stabilize the binding of Slit and Robo. Early studies have found that the Slit-Robo signaling pathway is the neuronal axon guidance factor, the neuronal migration-rejecting factor, and the inhibitor in leukocyte chemotaxis. It has been found that it plays an important role in cell migration, apoptosis, and angiogenesis in recent years.

Neutrophils in the infected and injured areas are important components of the inflammatory response. Neutrophil chemotaxis, migration, and adhesion are important processes of inflammation, and various chemokines and chemical inducers can regulate leukocyte chemotaxis. Robo1-Slit2 has been proven to be an endogenous inhibitor of leukocyte chemotaxis and inhibits the migration of neutrophils, lymphocytes, and macrophages in the inflammatory response.

Slit2 inhibits VEGF-induced wild-type vascular endothelial cell migration, duct formation, and increased permeability, thereby blocking angiogenesis and vascular leakage. The Slit-Robo signaling pathway controls the integrity of vascular endothelial cells, and Slit2 may become a new drug for the treatment of neovascularization and increased vascular permeability. Slit mediates Robo4 activation to form the paxillin-GIT1 (ArfGAP) complex, which inactivates Arf6 and ultimately enhances vascular stability. Slit2 also induces angiogenesis through activation of mTORC2 (mammalian target of rapamycin)-dependent Akt and Rac.

3.4.6 Sonic Hedgehog

Sonic hedgehog is an important signaling pathway involved in growth, development, and injury repair, but it is well studied in tumors. This pathway is also closely related to the biological function of the nervous system, which promotes neural development and regenerative axon guidance. In recent years, research on stress damage, especially in oxidative stress damage, has become a new hot spot.

3.4.6.1 The Hedgehog Signal Transduction Pathway and its Member

The Hedgehog (Hh) gene is found in drosophila. There are three Hedgehog signaling proteins in mammals: Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh), among which Shh is a comprehensive one. Ptch is a 12 transmembrane receptor and Hh signal transduction starts from Ptch. When there is no Hh signal, Ptch binds to Smo to prevent the movement of Smo to primary cilia and inhibit Smo activity, and downstream signaling pathway is inhibited. Ptch does not directly bind to Smo, but indirectly affects Smo by altering the distribution and concentration of certain small molecule inhibitors. Smo is a 7 transmembrane receptor protein in the Frizzle family and is a member of the G-protein-coupled receptor family. Its C-terminus is located in the cell, while the N-terminus located outside the cell has a glutathione-rich domain and a transmembrane structural fragment. SAG (Smo agonist) and cyclopamine (Smo inhibitor) bind to the extracellular N-terminal structure of Smo and play a role. Gli protein is a transcription factor in the Hh pathway. There are three subtypes: Gli1, Gli2, and Gli3. Gli1 plays a major role in transcriptional activation in the Hh pathway, Gli3 acts as transcriptional repression, and Gli2 has dual roles of transcriptional activation and inhibition. When Hh is activated, the Hh signal peptide binds to Ptch, which relieves Smo inhibition. Smo accumulates on the primary cilia in an active form, enters the cell and activates the transcription factor Gli to enter the nucleus, regulates the opening of downstream signaling pathway, and synthesizes downstream-related functional proteins such as N-myc and cyclin.

3.4.6.2 Hh Nonclassical Pathway

However, in recent years, there have been some reports on nonclassical Hh pathway. These non-classical pathways are mostly divided into two categories: independent of receptor Smo or independent of transcription factor Gli. A nonclassical pathway that does not require the receptor Smo: a nonclassical Hh pathway that promotes apoptosis and anti-tumor through the interaction between Ptch1 receptor and the cyclin B1. A nonclassical pathway that relies on the Smo receptor but does not require the transcription factor Gli: a nonclassical pathway that relies on Smo to activate the Rho family small molecules GTPases RhoA and Rac1, that relies on Smo to activate src to regulate axonal-directed nonclassical pathways, and that relies on Smo to regulate calcium signaling pathways and membrane function.

In addition to the above two categories, the study reports that during spinal nerve growth, Shh signaling dependent on Smo changes a classical Shh pathway that activates Gli1 transcriptional activity into a nonclassical Shh pathway that inhibits Gli1 transcriptional activity by regulating Ca^{2+}-dependent electrophysiological activity and CREB/PKA pathway. These nonclassical pathways are complementary to the complex Hh signaling pathways.

Previous studies have suggested that the Shh signaling pathway is only expressed in malignant keratinocytes in which the signaling pathway is activated, but not in normal keratinocytes, suggesting that specific regulation of the Shh signaling pathway only affects tumor cells, but not normal cells. Some scholars found that Shh can induce HaCaT keratinocytes to produce EGF. At the same time, EGF can in turn regulate the expression of Gli-1, the target gene of Shh signaling pathway in keratinocytes. This indicates that the Shh signaling pathway is closely related to the EGFR signaling pathway, which is a direct upstream pathway of the MEK/ERK pathway, further suggesting that the MEK/ERK signaling pathway may play an important role in the regulation of the metabolism of keratinocytes by the Shh signaling pathway. There are also many reports that the Shh signaling pathway plays an important regulatory role in the proliferation, apoptosis, and matrix penetration of normal keratinocytes. It is fully demonstrated that the Shh signaling pathway plays an important role in the proliferation and apoptosis of normal keratinocytes, but its molecular mechanism has not yet been elucidated.

Sonic hedgehog also has the effect of inducing angiogenesis, and the diameter of the formed blood vessels is large. Studies have shown that this is mainly related to Sonic hedgehog's ability to induce the expression of endothelial growth factor I and II, and its ability to rapidly activate C-Fes/PI3K pathway and gene transcription and regulate endothelial capillary formation, in which NO plays an important role. Some studies have found that in the animal model of diabetes, activation of the Hedgehog signaling pathway can partially improve the complications of diabetes. Studies by domestic scholars have found that the Shh signaling pathway was impaired in the skin tissue of diabetic mice, and the treatment of Shh protein on the wound surface can significantly promote the healing of diabetic skin wounds.

3.4.7 Other Signals

In the past, when people studied the molecular signaling mechanism of self-renewal, growth, and differentiation of cells (including stem cells), they paid more attention to the biochemical signals generated by chemical substances or solution media, and often ignored the influence of some physical factors or biomechanical signals. In recent years, some studies have begun to pay attention to the role of mechanical signals generated by the mechanical microenvironment inside and outside the cell and the mechanical force generated by cellular mechanical forces in controlling cell morphology and function. Biomechanical and mechanical signals are involved in the process of cell self-renewal and differentiation.

3.4.7.1 Extracellular Microenvironment and Biomechanics of Cells

Extracellular Microenvironment

Cell aggregation and intercellular connectivity are affected by the microenvironment of the extracellular matrix, which are mainly chemical soluble and physical signal networks composed of hydrated proteins and polysaccharides. The signal molecule specifically binds to the receptor on the cell surface, causing a series of complex intracellular signal responses, regulating the expression of the target gene, causing a change in the phenotype of the cell, which in turn leads to a series of biological reactions.

Biomechanical Effects of Cells

Cell Tension

The integrity structure of cell tension consists of a pressure-bearing member and a series of continuous tension members connected with each other. The stability of this structure depends on the maintenance of the internal structural integrity of cells, and it is also called tension integrity. Studies have shown that the structure of cells conforms to the principle of tension integrity, and the tension integrity of the cellular framework (CF) affects the shape and function of cells. In addition, the tension integrity of the cytoskeleton is a major determinant of cell deformation. Related studies have shown that DNA synthesis of flat cells are more vigorous than that of round cells, suggesting that cell deformation is an important part of information transmission. Under the action of mechanical stress, all components of the cytoskeleton undergo an overall rearrangement for dispersing tension and pressure, which will cause cell deformation and the regulation information of cell shape, is transmitted in the form of force. Therefore, the change of mechanical stress can affect the growth and biochemical properties of cells by changing the balance of forces in the cells and their skeletons.

Hydrostatic Pressure

For the Relatively static fluid state, due to its own gravity or other external force, there is a force perpendicular to the contact surface inside the fluid and between the fluid and the wall surface of the container, which is called the static pressure of the fluid. Current studies have shown that hydrostatic pressure plays an important role in regulating cartilage metabolism and maintaining the normal phenotype of extracellular matrix, in which static pressure stimulation reduces the synthesis of type II collagen and proteoglycans, while normal dynamic pressure stimulation promotes the synthesis of type II collagen and proteoglycans. Studies have found that the force that causes the cell shape to become round in mitosis depends not only on the cytoskeleton of actomyosin, but also on the cell's ability to respond to the change of "osmolality" (i.e., in order to represent the osmotic pressure of a solution, sometimes the molecular concentration of an isotonic nonelectrolyte solution is used, referred to as osmolality). Studies have shown that the force that causes the cell shape round is due to changes in osmotic pressure, and it is necessary for actomyosin cortex to resist the external forces to maintain cell rounding. The transient decomposition of the actomyosin cortex increases the cell volume, and the actomyosin cortex contraction stimulation causes a decrease in cell volume, indicating that the cells maintain the balance of osmotic pressure by intrinsically regulating the contraction of the actomyosin cortex. Thus, by modulating actomyosin-dependent surface tension locally and osmotic pressure globally, cells can control their own volume, morphology, and mechanical properties.

Fluid Shear Force

Some cells may be in a fluid flow environment for a long time, for example, endothelial cells and smooth muscle cells of blood vessels and lymphatic vessels, epithelial cells of the esophagus and intestinal tract, and osteoblasts and osteoclasts in bones. These cells are always exposed to the scour of blood, lymph, tissue fluids, or digested food debris, and the mechanical forces that such fluids exert on cells are called fluid shear forces. Studies have shown that fluid shear forces can regulate cell function. Fluid flow is currently considered to be divided into two main forms: laminar flow and turbulent flow. Recent studies have shown that fluid shear force is an important factor affecting tissue metabolism, especially bone tissue metabolism, and regulating bone cell function and morphology.

3.4.7.2 Effects of Biomechanical Force and Mechanical Signal Transduction on the Self-Renewal and Differentiation of Stem Cells

In the study of stem cells, how to maintain self-renewal of pluripotent stem cells is an important part of the research. In

addition, pluripotent stem cells can be induced or spontaneously differentiated into tissue-specific cells, such as nerve cells, skin cells, or muscle cells. But for a long time, researchers have added growth factors and small molecule compounds to maintain the pluripotency of pluripotent stem cells. Even so, in the long-term culture process, stem cells will enter different stages of differentiation, showing different gene expression pattern. Therefore, how to maintain the characteristics of pluripotent stem cells long-termly and effectively in vitro is still a subject worthy of further study.

Basement Hardness Affects the Proliferation and Differentiation Fate of Stem Cells

The importance of extracellular matrix sensing elasticity has been demonstrated in studies of fibroblasts, mesenchymal stem cells, and epidermal stem cells. Engler et al. found that different matrix hardness has different effects on the fate of cell growth. When the cells are cultured in a soft matrix similar to brain tissue, the cells show a neuronal phenotype; when the cells grow in a medium hardness matrix similar to muscle tissue, the cells show myogenic properties; and when the cells grow in a hard matrix similar to bone collagen, the cells show osteogenic phenotypes. Researchers at the University of Michigan in the United States found that stem cells exert a certain amount of force on the cell matrix, and this force may be related to cell differentiation. For example, stem cells grow in a hard matrix and finally differentiate into osteoblasts, while growing in a soft matrix differentiate into adipocytes. In addition, a team led by Professor Wang Ning, a Chinese-American scientist at the University of Illinois in the United States, found that mouse embryonic stem cells (mESCs) tend to adhere together to form round clones, but those cells contact hard culture dishes at the edge of the clones are relatively easy and rapid to differentiate. Based on this phenomenon, the researchers focused their attention on the biomechanical rather than biochemical studies of mESCs and found that cells cultured on soft gels showed greater homogeneity and pluripotency. Even in the absence of LIF, cells can be cultured continuously for more than 3 months. This study shows that reducing the elasticity of the matrix and cells helps the cells maintain a pluripotent state. However, the molecular mechanism of the change of extracellular matrix elasticity in inducing stem cell-specific differentiation has been a problem in the field of biomechanics.

Changes in the Shape of the Basement Affect the Proliferation and Differentiation of Stem Cells

The different shape of the bottom of the cell culture medium has an important influence on cell proliferation and differentiation. Nelson and Bissell found that cells proliferated fastest in the places with large tractive force, that is, the edge of the round dish and the four corners of the square dish. McBeath et al. found that mesenchymal stem cells cultured on a small substrate differentiated into adipocytes, whereas those cultured on a larger substrate differentiated into osteoblasts. In order to study the effects of basement geometry and biomechanics on the differentiation of mesenchymal stem cells, Li Zhenhan et al. found that the geometry and area of cell spreading were significantly associated with the proliferation, differentiation, and migration of bone marrow mesenchymal stem cells. When the cell spreading width was narrow, the proliferation of bone marrow mesenchymal stem cells could be inhibited; when the cell spreading area was limited, the cell migration was enhanced. Studies suggest that dexamethasone may play an important role in reducing cell proliferation and migration. In addition, the team further explored the effects of three biophysical parameters of matrix hardness, shape, and dimension on rat mesenchymal stem cells, indicating that matrix hardness or dimension plays a major role in proliferation and regulation of cells growing in harder, dimensionally unbalanced matrices. However, the matrix geometry plays an important role in controlling the shape and extension of the cells growing in the columnar matrix and forming globular rather than grooved cells. Studies have shown that although the softness of the matrix regulates rat mesenchymal stem cells to differentiate into bone cells or differentiate into neural cells, matrix geometry and dimensions also play a regulatory role in stem cell differentiation. Lü et al. used a polyacrylamide hydrogel to precisely process three kinds of topographical culture dishes (grooves, columns, and hexagons) to culture mouse embryonic stem cells and systematically analyze the morphology, proliferation, and dryness of the cells. Studies have shown that the topological topography of the substrate plays an important role in regulating the dryness of stem cells. The clones formed in the groove-like and cylindrical dishes are relatively flat, while the clones formed at the bottom of the hexagonal dish are more rounded.

Changes in Cell Morphology Affect Stem Cell Proliferation and Differentiation Fate

The shape and morphological characteristics are altered during cell differentiation. Studies have shown that cell morphology has an important relationship with cell proliferation, survival, and differentiation. Can changing cell morphology alter the differentiation fate of human mesenchymal stem cells? Early studies have shown that changes in cell morphology affect the activity of GTPase in the Rho family. The study found that RhoA is involved in the process of cell deformation, and changes in RhoA activity mediate the different differentiation of human mesenchymal stem cells. Inhibition of RhoA activity induces the production of adipocytes in human mesenchymal stem cells. Instead, continuous activation of RhoA can turn cells into osteoblasts. In addition, changes in cell morphology affect multiple regulatory

mechanisms of RhoA downstream signaling. Studies have shown that members of the Rho GTPase family need to be activated in lipid rafts, and this position will change with the change of cell shape. RhoA-ROCK (RhoA-dependent kinase)-tension signals affecting stem cell fate may be transduced by focal adhesion. Altering cell elongation will alter RhoA-mediated contraction of cytoskeleton, assembly of focal adhesions, and downstream signaling pathways of integrins. In summary, the interaction among cell morphology, biochemical signal, and skeletal contraction mentioned above demonstrates the importance of cell structure and mechanics in cell and tissue development, and it provides a molecular basis for cells and tissues to complete negative feedback regulation of stabilization mechanisms.

Effect of Biomechanical Force on Gene Expression and Differentiation Fate of Stem Cells

Fluid shear forces play an important role in the differentiation of embryonic stem cells and mesenchymal stem cells. In embryonic stem cell research, Yamamoto et al. found that compared with static culture, the number and proportion of cells in the S and G2 ~ M phases were significantly increased. In addition, shear forces significantly increased the protein level and RNA expression of vascular endothelial cell marker molecules FLK-1, FLT-1, VE (vascular endothelial)-cadherin, and PECAM (platelet endothelial cell adhesion molecular)-1. Adamo et al. have shown that fluid shear forces can promote hematopoiesis during embryogenesis. When mouse embryonic stem cells differentiate in vitro, the expression of Runx1 in CD41 and c-kit double-positive hematopoietic precursor cells increased under fluid shear stimulation. In addition, the elimination of NO signal may be one of the reasons why shear forces increases the ability of hematopoietic cell clone formation and increases the expression of hematopoietic marker molecules in mouse embryos. Toh and Voldman found that fluid shear specifically upregulated the ectodermal specific marker FGF5 (fibroblast growth factor 5). The mechanical perception of shear force in mouse embryonic stem cells may be mediated by extracellular heparin sulfate proteoglycans, thereby regulating FGF5 expression. In terms of mesenchymal stem cells, using DNA chip and reverse transcription real-time quantitative PCR analysis, Glossop and Cartmell found that different fluid shear strength and action time affected the expression of related genes in the mitogen-activated protein kinase (MAPK) signaling pathway of human bone mesenchymal stem cells (hBMSCs). Under stimulation of different shear strength and action time, different gene expression patterns appeared in the cells, but when comparing the cells with different mechanical strength and action time, it was found that regardless of the mechanical strength and action time, the shear force could consistently and significantly upregulate the expression of MAP3K8 and IL-1. This study suggests that MAP3K8 may be an important mediator of intracellular force transduction in hBMSCs. Other studies have shown that shear forces stimulate the phenotype of hMSCs to differentiate into osteoblasts without the induction of chemical factors. Horizontal tensile stress has an important influence on the differentiation of mesenchymal stem cells. The changes of mesenchymal stem cells after being stretched by uniaxial horizontal stress were detected by micro image beam. The results showed that horizontal stress caused an increase in the expression of the smooth muscle cell marker molecule calponin1, while the expression of the cartilage matrix marker molecule decreased. If the cells are placed in the vertical direction of stress stretching, the previous changes of gene expression disappear. These results indicate that mechanical stress plays an important role in gene expression and cell fate of mesenchymal stem cells. He Xueling et al. found that both osteogenic chemical inducers and stress stimulation can promote osteogenic differentiation of rat BMSCs, and they have synergistic effects. Lü et al. performed a kinetic study of lateral migration and transmembrane migration of human epidermal cells or human fibroblasts using a stretching device and a modified invasive chamber method. It was observed that when epidermal cells were cocultured with fibroblasts in a noncontact manner, epidermal cells had asymmetric migration. For example, the number of human epidermal cells which are distant from human fibroblasts or can migrate from bottom to top is significantly higher than those which are close to human fibroblasts or migrate from top to bottom. This asymmetric migration is primarily regulated by epidermal growth factor (EGF) secreted by fibroblasts. In addition, mechanical stretching can further affect the asymmetric migration of human epidermal cells by increasing EGF secretion of fibroblasts. High-frequency magnetic bead oscillation affects the proliferation and differentiation of stem cells. Chowdhury et al. placed micron-sized magnetic beads on the surface of embryonic stem cells and then applied a tiny oscillating magnetic field to oscillate the magnetic beads back and forth in the magnetic field. Studies have found that this continuous oscillation can stimulate the production of internal forces, such as the periodic movement of myosin. They also found that mouse embryonic stem cells are softer than other types of cells, and more sensitive to local periodic forces. A small local force can alter the expression of Oct3/4 genes in a single embryonic stem cell, and the expression of other genes can also be altered.

Effects of Mechanical Effects Produced by Rotational Culture on Stem Cell Proliferation and Differentiation

In recent years, it has been reported that the mechanical effects of rotation culture can affect the proliferation and differentiation of stem cells. Yuge et al. found that the cells expanded 14-fold after 1 week of culture, while normal gravity culture only expanded 4 times. In addition, cells expanded

in a 3D-clinostat have the ability to differentiate into hyaline cartilage in vivo. In contrast, cells cultured under normal gravity lose this property. The study suggests that a 3D rotation culture environment may be an important tool for expanding stem cells, and that expanded cells can avoid some adverse effects after cell transplantation. Kawahara et al. found that in the absence of feeder layer, serum, and LIF, mouse embryonic stem cells can still maintain dry growth, and expanded embryonic stem cells has the potential to differentiate into three germ layers. A rotary bioreactor developed by the National Aeronautics and Space Administration (NASA) was used to culture human foreskin-derived epidermal stem cells. The results showed that under the 3D rotation culture condition, the proliferation of human epidermal stem cells was enhanced, and the expression of the stem cells terminal differentiation marker molecule involucrin was reduced. The expanded cells form a compact 3D structure. In addition, in the study of mouse embryonic stem cell differentiation, it was found that rotary suspension culture promoted cell differentiation into mesendoderm, and the main mechanism was upregulation of Wnt/β-catenin signaling pathway. Although the mechanical effects of rotation culture can affect cell proliferation and differentiation, the exact mechanism remains unclear and needs further study.

3.4.7.3 Possible Mechanisms of Biomechanical Stress Regulating Stem Cells

The process in which cells sense the elasticity of ECM is the process in which cells transform the perceived mechanical stimuli into biochemical information. This process is called cellular mechanical signal transduction. The cells adhere to ECM through the adhesion spots, which are composed of a series of signaling molecules and are connected to the actin cytoskeleton. These signaling molecules can produce conformational changes under mechanical stimulation, triggering a series of molecular events such as kinase activation, phosphorylation site exposure, intracellular transport of signaling molecules, and changes in receptor ligand binding strength.

Integrin Activity and Mechanical Regulation of Signals

More and more studies have shown that a key molecular mechanism of cellular stress perception is the deformation of integrins, focal adhesion proteins, and other related structural proteins. Integrin has the function of mechanical stress transduction, which focuses on the integrin focused adhesion zone and acts to convert mechanical signals into chemical signals. Friedland et al. reported that α5β1-integrin is a switch that Myosin II causes cytoskeletal stress fibers to be in a state of relax and tension. There is some evidence that α-actin, which mediates actin filaments and the cytoplasmic portion of integrin, is a key molecular structure that interferes with the formation of actin stress fibers and blocks signaling transmission to the nucleus. G protein is a key signal transduction molecule. The large G protein is anchored to the cell membrane by lipid modification of amino acid residues on subunits, thus providing a structural basis for receiving the structural signals of cell membrane. The small G protein receives extracellular mechanical signals through the extracellular matrix-integrin-cytoskeleton. Recently, Grashoff et al. have demonstrated that the recovery of focal adhesions in living cells and the transmission of force between focal adhesions are necessary for the stabilization of focal adhesions under stress conditions by using the tension biosensor of focal adhesions. Therefore, integrin activity and signaling mechanical regulation may determine the fate of stem cells.

The Role of RhoA/Rho-Kinase (ROCK) Signaling Pathway

RhoA is a GTP enzyme located in the cell. ROCK is an effector molecule downstream of RhoA that is activated by activated RhoA. ROCK phosphorylates myosin light chain kinase (MLCK) and myosin phosphatase to inhibit the activity. This plays a very important role in maintaining the integrity of the cytoskeletal tension fibers, increasing the stretchability of actin tension fibers, and regulating cell morphology, adhesion, and movement. Although RhoA plays an important role in the mechanical response of cells, its molecular mechanism has not been revealed. Guilluy et al. found that tension stimulated the activation of these two molecules and recruited them to the adhesion complex via integrin. Furthermore, the results revealed that LARG is activated by Src family kinase Fyn. The enhancement of GEF-H1 activity is catalyzed by the downstream cascade of extracellular-regulated protein kinases (ERK), including focal adhesion kinase (FAK) and rat sarcoma (Ras).

The Function of Mechanical Signal Super Long-Distance Transduction

Short-distance mechanical transduction and mechanism in living cells have been well studied. Force and energy can cross-membrane through the integrin, adhesion proteins, binding focal adhesions, and cytoskeletal networks to connect the nucleus, nuclear internal scaffold, and circular chromatin to signal transduction. The speed and precision of this intracellular mechanical signal response can be adjusted by altering the prestress of the cytoskeleton. Prestress changes can control the stiffness of the cytoskeletal microfilaments under tension, such as actin stress fibers and intermediate fibers with large span in the cytoplasm. The force acting on the nucleus may cause morphological changes, folding and kinetic changes of specific load-bearing molecules in the nucleus, and may also alter the high-level structure of chromatin, which may affect the self-assembly of nuclear proteins, gene transcription, DNA replication, and RNA

processing. This long-distance transduction of mechanical signals in living cells is faster and more efficient than biochemical signal transduction generated by diffusion. Long-distance transduction of mechanical signals also helps to understand how mechanical forces alter the activity of multiple molecules at different locations in the cytoplasm and nucleus. This cellular response is critical for regulating cell behavior, tissue development, and the fate of stem cells.

The Role of Myosin II Regulating Stem Cells

Non-muscle myosin II (NMM II) is a class of actin-binding proteins that regulate cell contraction. Among the three different NMM II subtypes identified, NMM II A and NMM II B are found in almost all higher organisms. Knockout of NMM II A or NMM II B leads to fetal death in mice. NMM II is essential for early embryo development. The important role of NMM II in mechanical regulation of stem cells has been reported. Matrix elasticity and cell morphology determine the mesenchymal stem cell lineage, which depends on NMM II, and its specific molecular mechanism is still unclear.

E-cadherin is a key target molecule of NMM II A in human embryonic stem cells. NMM II-induced contraction is not only a function of reading extracellular signals, but also plays an important role in determining the production and transduction of stem cell signals.

With the deepening of research on the regulation of cell structure and function by mechanical stress in recent years, the response of cells to stress-induced strain and the mechanism of mechanical transduction have gradually been recognized. However, there are still many unknown details to be explored in this field, such as the dose-effect relationship between stress size, frequency and stem cell proliferation, differentiation, apoptosis, the effect of stress on the biological behavior of tissue-repaired stem cells, and the regulation of intracellular mechanical signal transduction and gene expression under stress. Therefore, there will be futher study on the relationship between mechanical stress, mechanical transduction pathway and cells, and explore a mechanical-biological coupling law of mechanical effects on the life activities of cells, which will help to understand the role of force in the pathogenesis and wound repair process of certain diseases and help to adopt new and more effective coping strategies for these mechanisms and processes. It is also important for the research of tissue engineering, gene therapy, stem cell regenerative medicine, space life, and space medicine.

AGEs-RAGE Signaling Pathway

Diabetic wound healing is a complex process of multifactor regulation such as inflammatory cells, repair cells, and cytokines. Abnormalities in any part can lead to difficult healing of wounds. Advanced glycosylation end products (AGEs) and related cell signaling pathways are research hotspots in the study of the mechanism of difficult wound healing in diabetic wounds.

AGEs and their receptors, RAGEAGEs, are a group of stable end products produced by condensation, rearrangement, cleavage, oxidative modification, and other complex nonenzymatic glycosylation reactions of various intermediates. Normal humans also have AGEs, but their formation takes several weeks or even months, and the production is very low. However, when protein half-life is long, protein turnover is delayed, persistent hyperglycemia (such as diabetes) and active carbonyl levels are elevated, AGEs will significantly accelerate synthesis, resulting in more production of AGEs than clearance, accumulation in the body, damaging cells and tissues. Persistent hyperglycemia causes nonenzymatic glycosylation of many structural proteins, functional proteins, and nucleic acid proteins in the human body, ultimately resulting in irreversible AGEs. The receptor pathway is the main pathway for the action of AGEs. Currently known AGEs receptors include RAGE, macrophage scavenger receptors I and II, oligosaccharyltransferase 48, and galactose-binding protein-3. RAGE is the most studied and in-depth one. RAGE is a polypeptide with a unique amino acid end sequence and a molecular weight of about 35 ku. It is a multi-ligand member of the immunoglobulin superfamily in cell surface molecules. Many cells, such as endothelial cells, monocytes-macrophages, and vascular smooth muscle cells, have RAGE expression on the surface. Therefore, these cells also act as sites for the physiological and pathological effects of AGEs and RAGE interactions. In the normal physiological state of mature individuals, the level of RAGE expression in cells is low. When diabetes, inflammation, and so on cause cells to be stressed, RAGE can be highly expressed.

Classical pathophysiological studies have shown that spontaneous ulcers or difficult wound healing in diabetic patients are closely related to angioneuropathy caused by disorders of glucose metabolism. However, from the perspective of mechanism research, angioneuropathy is the pathological outcome of the diabetes disease, and it is not the starting point of difficult wound healing. Studies have shown that in the case of undamaged skin tissue of diabetic patients, there have been changes in histology and cell biological behavior, which is a kind of “hidden damage” that does not cause skin tissue integrity and continuity damage.

Although this damage is endogenous, it does not cause visible damage to skin defects or fractures, changes in histology and cellular function may increase the vulnerability of skin tissue to exogenous damage. The “hidden damage” is caused by the high glucose environment of local skin tissue and the accumulation of AGEs, resulting in the cell or matrix dysfunction of the skin tissue. Therefore, the production and accumulation of AGEs is an important pathological basis for the damage of multiple tissues and organs such as nerves and

blood vessels. Vascular endothelial cells and fibroblasts are the main cells involved in repair during wound healing. The integrity of their structure and function is the main influencing factor of diabetic wound healing. Under normal conditions, endothelial cells express only a small amount of RAGE; in diabetic state, with the increase of AGEs in circulating and subendothelial matrix, the expression of RAGE in endothelial cells is enhanced and the interaction of AGEs with RAGE changes the function of endothelial cells. AGEs can significantly affect the morphology and proliferation of fibroblasts, inhibit the ability of fibroblasts to synthesize collagen, and affect wound healing. Studies have shown that AGEs exert biological effects primarily through two pathways: the non-receptor pathway (direct damage) and the receptor pathway. Non-receptor pathway: AGEs alter their structural functions by direct modification of proteins, lipids, nucleic acids, and the like. Receptor pathway: AGEs initiates a series of receptor signal transduction pathways by binding to RAGE, a specific receptor on cell membranes such as endothelial cells, to stimulate the formation and release of various cytokines, leading to pathological changes in the body's nerves and blood vessels. Among them, the receptor pathway is the main pathway of AGEs action, playing a more important role in the damage caused by AGEs.

(a) The combination of AGEs and RAGE promotes the increase of ROS production and induces oxidative stress. Abnormal oxidative stress leads to abnormal wound healing in diabetic skin. During wound healing, AGEs-RAGE effect is an important factor influencing the level of oxidative stress in diabetic wounds. The interaction of AGEs with its cell surface receptor RAGE is mediated by reduced coenzyme II (NADPH) oxidase, which increases ROS production, produces oxygen free radicals, causes cell membrane superoxide, impairs membrane protein function that maintains ion homeostasis, thereby causing calcium influx, increase of the intracellular calcium ion concentration, and finally lead to abnormal cell structure, function, metabolism, and induce oxidative stress reaction. In addition, when the ROS is excessive and exceeds the antioxidant capacity of the cells, it can directly activate or damage certain proteins, nucleic acids, lipids, etc. in the cells, affecting the function and integrity of the cells. Since ROS is produced in the mitochondria, mitochondria are most vulnerable. Oxidative stress promotes mitochondrial oxidative phosphorylation and also causes the formation of a large amount of peroxide, thereby inhibiting glyceraldehyde-3-phosphate dehydrogenase and causing accumulation of glycolysis intermediates. These intermediates further enhance the activity of aldose reductase and protein kinase C and in turn promote the production of AGEs, leading to nerve fiber dysfunction and damage.

(b) Activation of the nuclear transcription factor (NF-κB), triggering a cascade reaction that affects the healing of diabetic wound tissue. NF-κB is present in a variety of cells and is involved in various physiological and pathological processes such as immune regulation and inflammatory cell proliferation. NF-κB binds to the B cell-specific nuclear protein on the immunoglobulin κ light chain gene enhancer, exists in many types of cells, and controls the expression of various genes. The enhancers of these genes all contain binding site of NF-κB, which are the target genes regulated by NF-κB, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), endothelial cell adhesion molecule (ECAM-I), tissue factor (TF), monocyte chemoattractant protein-1 (MCP-1), nitric oxide synthase (NOS), and angiotensinogen gene. Activated NF-κB initiates transcription of these target genes, triggering abnormal inflammatory responses and autoimmune responses. In vivo, the activation-inducing response of NF-κB is transient and the system returns to baseline within a few hours. Diabetic patients have abnormal sustained NF-κB activation, and ligand-stimulatory RAGE activation can act as a switch to turn NF-κB short-term activation into long-term activation. AGEs interact with RAGE to cause oxidative stress and activate NF-κB by activating the proto-oncogene ras-encoded p38ms and mitogen-activated protein (MAP) kinase pathways. Activation of NF-κB stimulates the expression of numerous target genes, such as TF, P21, Has, and extracellular signal-regulated kinase 1/2 (ERK1/2), and the expression of RAGE. Activation of these pro-oxidative genes can increase the secretion of nitric oxide (NO), interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), monocyte clone stimulating factor, VCAM-1, and ICAM-1. The release of a large number of pro-inflammatory cytokines (IL-6, TNF-α), growth factors (transforming growth factor-β, IGF), and adhesion molecules (VCAM-1, ICAM-1) can trigger uncontrolled systemic inflammatory cascade, forming a vicious circle, causing persistent cell damage and dysfunction, ultimately leading to the development of tissue damage.

The AGEs-RAGE signaling pathway is closely related to the repair of diabetic refractory wounds, involving many cytokines, oxidative stress, nerves, blood vessels, and many other aspects. The AGEs-RAGE signaling pathway is the initial stage of invisible lesions in diabetic wounds. If the upstream part related to the mechanism of difficult healing wounds can be intervened to terminate the occurrence or development of subsequent effects, it can provide effective and feasible preventive treatment. It is of great significance to improve the cure rate of difficult healing

wounds, and also provides a solid theoretical basis for drug target intervention.

In summary, wound repair is a complex process involving many growth factors/cytokines and their signal transduction pathways. These pathways and growth factors/cytokines regulate each other to promote wound healing. This is a complicated process, and there are still many mechanisms that we need to explore.

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