Regenerative Medicine in Otolaryngology

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Editor

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Preface

The main objective of this issue is to introduce newly developed treatment methods using regenerative medicine in the field of otolaryngology. Most of the studies undertaken have been performed in the Department of Otolaryngology, Head and Neck Surgery of Kyoto University and related hospitals in Japan.

We have already published *Regenerative Medicine for the Inner Ear*, which summarized possible novel therapeutic strategies for the treatment of inner ear diseases, especially using regenerative medicine.

In this issue, the therapeutic target organs are the external ear, middle ear, tympanic membrane, oral organs, larynx, trachea, and other organs in the field of otolaryngology.

We have used several kinds of neurotrophic factors, cell replacement and transplantation method (in situ), tissue engineering method, and other methods related to regenerative medicine. Some are already clinically applied, and others are still in the animal experimental level. Most of the issues proposed and discussed in this book are still controversial; however, it is my earnest hope that this issue will serve as a useful guidance and understanding of the regenerative medicine in the field of otolaryngology.

I would like to thank all the authors for taking their precious time and tireless effort in contributing their findings to making the compilation of this issue possible. Effort and patience on the part of Springer Publishing in publishing this issue is much appreciated.

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Chapter 1
Current Situation of Regenerative Medicine

Shin-ichi Kanemaru

Abstract Regenerative medicine has made remarkable progress. This has been supported by the development of tissue engineering, which was a combination of medicine and engineering. Tissue engineering applies the principles and methods of engineering, material science, and cell and molecular biology toward the development of viable substitutes that restore, maintain, or improve the function of human tissues.

According to the theories of tissue engineering, tissues and organs can be regenerated by manipulating three elements: cells, scaffolds, and regulation factors. In this field, cells mean stem cells that possess both capabilities of self-renewal and differentiation into various tissue-specific cells. Stem cells are divided into three groups: embryonic stem cells (ES cells), somatic stem cells, and induced pluripotent stem (iPS) cells. Although ES cells are the best cell-source having the omnipotency to generate all tissues, ethical problems and rejections by the immune system remain to be resolved. On the other hand, somatic stem cells are promising cell-sources because they are free of the above problems. Actually, almost all clinical applications of regenerative medicine have been performed using somatic stem cells or progenitor cells.

Mesenchymal stem cells (MSCs) belonging to somatic stem cells are defined as pluripotent progenitor cells with the ability to generate bone, cartilage, muscle, tendon, ligament, and fat. Moreover, in some kind of conditions, MSCs can be differentiated into another lineage: nerve, epithelium, and so on. These properties have generated great interest in the potential use of MSCs to replace damaged tissues. Mesenchymal stem cells could be cultured to expand their numbers or after or seeded in/on shaped biomimetic scaffold to generate appropriate tissue constructs, then transplanted to the injured site.

The iPS cells have been considered to be comparable to ES cells and are promising cell-sources as substitutes for ES cells. Generation of iPS cells has dramatically changed the landscape of stem cell research and its future clinical application.

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Especially, it is possible that novel drug discovery using iPS cell-based disease models/toxicity screening can be useful in filling the gap between animal models and clinical trials.

A scaffold that sustains cells is necessary to regenerate injured tissues and organs. Owing to the development of biotechnology and polymer chemistry, we can create more desirable biomaterial scaffolds with appropriate mechanical properties that can be modified to incorporate biological activity, such as growth factors and structural adhesive proteins. It is no exaggeration to say that successful regeneration of tissues and organs depends on how to establish an appropriate scaffold in the injured site.

Regulation factors also play an important role in tissue regeneration. They can induce angiogenesis, which promotes a sufficient supply of oxygen and nutrients to effectively maintain the biological functions of cells transplanted for organ substitution. Therefore, if we do not understand and manipulate the complex relationships among cells, scaffolds, and regulation factors, we cannot repair and/or regenerate tissues and organs.

This chapter discusses the basic principles of tissue engineering and introduces current situations of regenerative medicines.

Keywords Regenerative medicine • Tissue engineering • ES cells • Mesenchymal stem cells • iPS cells • Scaffold • Regulation factor

1.1 Introduction

Regenerative medicine is defined as the field of study in which irreversibly damaged tissues and organs are reconstructed by artificial control of regeneration, a vital biological phenomenon. This regeneration is achieved by induction of cell differentiation and growth.

When an organism injures a part of its body, it has a regenerative ability to return the injured area into its original state. It is one of the basic phenomena of life. When tissue or an organ is damaged, spontaneous healing occurs depending on the severity of the impairment. Sometimes the damaged tissue or organ fully regenerates to its original state, and other times it does not because of deformity or degeneration. That is, there is a limit to the natural regenerative ability of an organism. The regenerative ability varies with tissue and organ. For example, when one half of the liver is resected, it can regenerate almost to its original size in only a few months. The regenerative ability also greatly varies by the type of animal. It is well known that newts and lizards can completely regenerate severed tails and legs. In general, tissues and organs with more vigorous cell division have more vigorous regenerative activity, and lower animals have greater regenerative abilities. Regenerative

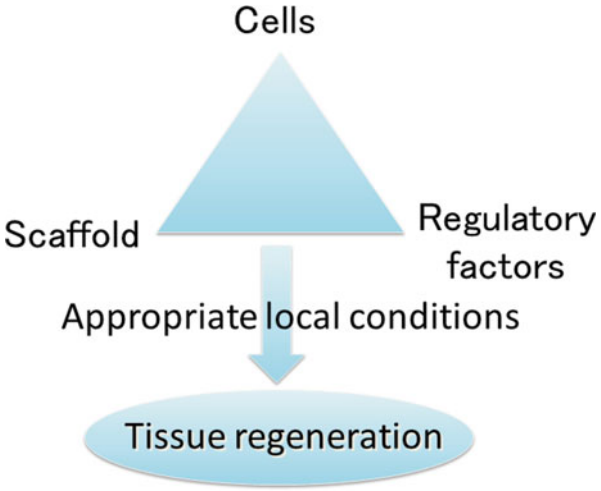

Fig. 1.1 Tissue Engineering Triad

medicine involves research of developmental process and regeneration in various animals. It examines the progress of regeneration in tissues and organs in which regeneration normally does not occur, and examines healing to the original state. Regenerative therapy is a result of regenerative medicine research reaching the point of clinical application as treatment.

Regenerative medicine originated from research to develop artificial organs in the 1950s, mainly in the West. Research and development were conducted on materials with high tissue compatibility for artificial organ transplantation into a living body. Such efforts led to a new field of medicine, which combines engineering, called "tissue engineering." Tissue engineering [1] can be said to be the foundation of regenerative medicine and is also called "(tissue) regenerative medical engineering." Regeneration of tissues and organs require cells, scaffold, and regulatory factors in an appropriate environment, as shown in Fig. 1.1. Cells form the basis of regeneration, a scaffold is needed for differentiation and growth of cells, and regulatory factors control their differentiation and growth. Cells, scaffold, and regulatory factors are called the "3 elements of tissue engineering." The key to regeneration lies in how to use these elements, and a major factor in the success of regeneration is creation of a favorable regenerative environment. Regenerative medicine has advanced due to recent remarkable progress in tissue engineering, molecular biology, and developmental genetics. In particular, research in stem cells has made major advancements. The selection of cells has increased further with the generation of induced pluripotent stem (iPS) cells. However, there are still issues that must be resolved before these cells can gain wide clinical application. There are medical issues involving the safety of cell transplantation, source of cells for harvest, and immunological rejection. There are also many ethical and social issues. Therefore, results of stem cell research have been clinically applied in only certain fields. It is

presently unknown whether regenerative therapy will become established as a major modality of medical treatment. However, many research studies have opened new avenues to tissue and organ regeneration. This paper reviews research methods and concepts that are the basis of regenerative therapy, focusing on the craniocervical region.

1.2 Approaches to Research in Regenerative Medicine

There are several approaches to research in regenerative medicine but this paper will categorize them into in vitro and in vivo research. Tissue engineering began in the 1970s and its concept was established in the early 1990s [1–3]. The original concept was based on the in vitro approach. In other words, the three aforementioned elements of tissue engineering were prepared and cultured in vitro. The target tissue was regenerated ex vivo up to some stage and then implanted in the living body. This method can be considered the ultimate regeneration method because mass production will be possible if the process is established. Presently, this method has been implemented only for regeneration of skin [4] and cornea [5]. It is difficult to use this method in some organs, particularly structurally large solid organs (such as liver, kidney, and pancreas). Although there is research in hybrid artificial organs using xenogeneic and allogeneic functional cells, such organs have not been used in clinical application. Okano et al. developed cell sheet engineering [6, 7] using temperature-responsive polymers. Cell sheet engineering is a field of study which enables regeneration of organ with local damage and has wide-ranging applications. Conventional methods posed many difficulties for tissue repair, including the need for enzymatic treatment to collect cultured cells, resulting in the destruction of adhesion between cells and therefore of the network of cells. Thus, cell clusters had to be implanted in pieces, and the cells needed to reconstruct a network at the recipient site. When cells are cultured as a cell sheet, tissue repair can be performed by affixing the sheet to the injury site. The application of this method has been studied for tissue repair of cornea, periodontal tissue, esophagus, lung, heart, liver, and pancreas.

Regeneration using an in vivo approach involves direct implantation of the aforementioned three elements in a living body. This method is divided into two categories depending on the size (presence or absence) of the morphologically damaged site. In other words, if there is a small or no defect, the scaffold still remains. Therefore, it is unnecessary to implant extracellular matrix as a scaffold. Thus, only implantation of cells, or implantation of cells and administration of regulatory factors, is needed. This situation applies to bone marrow transplant [8] and transplant of embryonic nigral cells (dopamine-producing cells) for Parkinson's disease [9, 10]. It also applies to cell transplantation for myocardial infarction [11]. If there is a large defect, regeneration is required not only at the cellular level but also at the level of organ structure. That is, a scaffold is needed for cell growth as well as for structure to maintain morphology of the organ itself. For example, tracheal morphology is

maintained in tracheal regeneration using the basic structure of a polypropylene Marlex mesh reinforced with a spiral of polypropylene wire. This basic structure is coated with collagen sponge as the artificial extracellular matrix [12].

1.3 Field Theory and In Situ Tissue Engineering

As mentioned previously, a living body has a natural ability for regeneration. However, if this ability is inhibited due to some reason, then regeneration ceases. For example, a living body responds to an injury to a tissue or organ, resulting in a partial defect. The injury triggers a signal for regeneration, and simultaneously the following urgent responses occur; through these responses, the body tries to protect itself by quickly isolating the injured site from its surroundings:

1. Hemostasis by platelet aggregation and vasoconstriction, and phagocytosis of necrotic tissue by macrophage
2. Contraction of granulation tissue composed mainly of collagen secreted by fibroblasts
3. Changing of granulation tissue into scar tissue, and the resulting stability of the wound

These responses degrade the scaffold for regeneration depending on the site and depth of the defect. As a result, regeneration of tissue that normally occurs does not. In such a case, if cells of the target tissue or organ still remain, then regeneration can proceed by securing a space for the scaffold before invasion of fibrotic connective tissue. That is, while isolating the defect site from its surroundings, an extracellular matrix is supplied from an external source to secure a scaffold for regeneration. If the amount of cells is insufficient, then cells are also supplied from an external source. In any case, it is essential for regeneration to have a site with a favorable environment, namely a rich blood supply, and to have a certain amount of time. In other words, it is important to create an optimal regenerative environment that allows the damaged tissue or organ to show its maximal, natural regenerative ability. The optimal environment enables regeneration to proceed in accordance to the situation. This concept is the "field theory." The method of in situ tissue engineering is based on this "field theory." In this method, it is not always necessary for all three aforementioned elements (cells, scaffold, and regulatory factors) to be supplied from external sources. An external source can supply only one or two elements to the regeneration site in the living body. For example, an external source can supply scaffold alone, scaffold and cells, or cells and regulatory factors. The remaining element or elements can be provided by the body itself, enabling tissue regeneration [12–15]. However, it does not mean that all tissues and organs can be regenerated by this method, and this method is indicated only in local regeneration of specific organs. In many cases, regenerative therapy that is currently performed is based on this field theory.

1.4 Cells

Cells are the main players in regenerative medicine. Since cells are the basis of tissue regeneration, they are essential in regeneration. Cell transplantation is not always necessary if the amount of cells is sufficient at the site for regeneration. However, if one cannot expect sufficient cells to be supplied by their surroundings, then one must administer the cells supplied by an external source. The methods of administration are local administration and systemic administration. Local administration includes a method in which the cells are cultured in vitro and tissue regeneration is allowed to proceed to a certain degree before implantation into the body. The cells for implantation must be cells that actively grow in the living body and perform tissue regeneration. If cells are completely mature and cannot grow, they cannot fulfill this role. Therefore, cells suitable for implantation are stem cells and tissue stem cells. Stem cells have two basic characteristics, but there are various types of stem cells, differing in the degree of differentiation:

1. Ability to differentiate into two or more different types of cells
2. Self-renewal ability

Embryonic stem (ES) cells are the most upstream stem cells and are totipotent. In principle, a single ES cell can give rise to all cells, and thereby all tissues of the organism. Somatic stem cells (tissue stem cells) are slightly more downstream compared to ES cells and have a considerably clearer direction of differentiation.

1.4.1 ES Cells

Human ES cells are cultured from tissue called the inner cell mass of the blastocyst (5 days after fertilization), which is formed by division of a fertilized egg. In 1998, Thomson et al. successfully isolated human ES cells [16]. It is very difficult to maintain human ES cells compared with ES cells of other animals such as mice. When ES cells are maintained, the cells do not merely grow but they maintain the totipotency of the original ES cells as they continue to be cultured. ES cells divide spontaneously at a certain rate under normal culture conditions. Therefore, they cannot be maintained in their undifferentiated state [15]. A cytokine that maintains the undifferentiated state of ES cells, called a leukemia inhibitory factor (LIF), has been identified in mice. However, this type of factor has not yet been discovered in humans. Presently, undifferentiated human ES cells can be maintained by culturing them on a feeder cell layer or by culturing them in laminin-coated cultureware containing culture supernatant of feeder cells [17]. Cells such as mouse embryonic fibroblasts (MEFs) have been used as feeder cells.

When ES cells are used for tissue regeneration, they give rise to teratoma if the cells are transplanted as they are. Therefore, induction of differentiation must be performed to a certain extent, and subsequently the target cells are selectively

harvested, grown, and eventually transplanted. In mice, the methods for induction of differentiation include a method that involves embryoid body (EB) formation and a method that involves coculturing with stromal cells. ES cells have a characteristic of adhering to walls of cultureware. However, these cells form a mass when grown in suspension culture that does not allow them to adhere to cultureware. The method of EB formation uses such a tendency of ES cells. Differentiation in this mass spontaneously progresses. This mass includes tissue progenitor cells of all germ layer lineages (ectoderm, mesoderm, and endoderm) and stem cells [18]. This mass is called an embryoid body, which differentiates into specific cells after stimulation with growth factors and cytokines. These cells are selectively grown and used in cell transplantation [19, 20]. The method of coculturing with stromal cells involves bone marrow stromal cells that maintain differentiation and growth of hematopoietic cells. When ES cells are cocultured with these stromal cells, induction of differentiation can occur in a certain direction [21, 22]. This technique to induce differentiation is still insufficient for humans. However, there have been reports on induction of differentiation of human ES cells into cardiomyocytes [23], neural stem cells [21], and vascular endothelial cells [24]. In the future, techniques will likely be established to produce progenitor cells and tissue stem cells for various organs.

1.4.2 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a type of somatic stem cells. MSCs together with vascular and hematopoietic stem cells were discovered in bone marrow stroma. Human MSCs are known to express the following surface protein markers: CD106, CD166, CD29, CD105 (SH2), CD73 (SH3/4), CD44, CD90 (Thy-1), CD71, and Stro-1. However, MSCs are negative for CD31, CD18, CD56, CD45, CD34, CD14, and CD11.

Mesenchymal stem cells can be isolated from bone marrow, which is collected by bone marrow aspiration. The primary culture is grown from the bone marrow aspirate and the suspension cells are removed to obtain cells that adhere to and grow on the bottom surface of the cultureware. The resulting cells are bone marrow stromal cells (BSCs). These cells support the microenvironment in the bone marrow and control the differentiation and growth of hematopoietic cells. MSCs are included in BSCs. MSCs can differentiate into appropriate progenitor cells, which in turn differentiate into mesodermal tissues such as bone, cartilage, muscle, adipose tissue, tendon, ligament, connective tissue, and cardiac muscle [25, 26]. Depending on the conditions of induction, MSCs also have the ability to differentiate into ectodermal tissue such as neurons [27] and epithelial cells, or into endodermal cells. MSCs are present in multiple tissues in the body. In recent years, MSCs have been isolated from various sites (other than from bone marrow) such as adipose tissue, amniotic fluid, periosteum, and fetal tissue [28, 29]. MSCs are known to differentiate into various cells, including cells of mesodermal tissues, in response to a trigger such as trauma, and there are reports that MSCs are involved in tissue regeneration [29–31].

It has also been reported that when bone-marrow-derived mesenchymal stem cells are implanted into mice, the cells differentiate into various organs and become engrafted [27]. These findings indicate that MSCs derived from bone marrow or adipose tissue can be selectively grown, cultured under certain conditions, and induced to differentiate into specific tissue, enabling tissue regeneration. In addition, successful regeneration has been reported in which multipotency of MSC was used and cells were directly implanted in the injured tissue to simultaneously regenerate multiple tissues (vocal cords) [32].

The greatest advantage of mesenchymal stem cells is that they are easy to harvest from autologous tissue. These cells can be said to be the easiest stem cells to use in local regeneration of injured tissue.

1.4.3 iPS Cells

In 2006, Yamanaka and Takashi et al. created undifferentiated pluripotent cells, iPS cells. They generated iPS cells from mouse skin fibroblasts by the retroviral transduction of four genes (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) called Yamanaka factors [33]. The iPS cells have been considered to be comparable to ES cells. Subsequently, Yamanaka et al. also generated human iPS cells [34]. Thereafter research on iPS cells has rapidly advanced. These cells have been generated by insertion of different types of genes, the use of different number of genes, and the use of different methods for gene transduction. As a result, the efficiency of iPS cell generation has improved compared with the initial method. Malignant transformation has been the greatest problem when the cancer-related gene *c-Myc* has been used. A method was developed which used the *Glis1* gene and all Yamanaka factors, except for *c-Myc*. This method avoided the problem of *c-Myc*-associated malignant transformation and improved the efficiency of iPS cell generation [35].

The greatest significance of successful iPS cell generation is that it broke the preconceived notion that "completely differentiated somatic cells cannot be reprogrammed." Since the generation of iPS cells, there has been advancement in research on their clinical application. In particular, Takahashi et al. has successfully induced differentiation of autologous iPS cells into retinal pigment epithelial cells. This effort was the first clinical application of iPS cells, and was used for the treatment of age-related macular degeneration (wet type). The study used a cell sheet composed of morphologically and functionally mature retinal pigment epithelial cells, which were derived from human iPS cells [36]. There has been much anticipation in the field of drug discovery, involving disease models using iPS because results can be obtained quickly.

A current approach to clinical application of new drugs involves finding drug candidates that target the molecules causing the disease, performing molecular modifications, and further narrowing down the candidates. This process uses mainly animal-derived cells. However, the use of human iPS cells enables researchers to narrow down drug candidates to effective compounds at the initial stage of this

process and enables faster drug development. In addition, the use of human iPS cells enables accurate and efficient evaluation of the effectiveness and safety of the drug candidates. For example, studies have already begun using cardiomyocytes generated from iPS cells to lower the risk of side effects. There is also a report on anti-amyloid-beta drug screening using human iPS-derived neurons for the treatment of Alzheimer's disease. Amyloid-beta is a protein considered to be the causative agent of Alzheimer's disease, and this screening detects the accumulation of amyloid-beta [37]. More applications of iPS cells are expected in the future [38].

1.5 Problems of Cell Transplantation

As mentioned above, if stem cells are used in regenerative therapy, it is necessary to use appropriate cells as a source depending on which tissue needs to be regenerated. However, not all somatic stem cells and progenitor cells have been discovered. In the preliminary step of cell transplantation, it is generally necessary to harvest the target cells and allow them to differentiate and grow to generate the required amount of cells.

The differentiation and proliferation potential is more inferior in the existing somatic stem cells and progenitor cells than in ES cells. Thus, it is difficult to obtain sufficient amounts of somatic stem cells and progenitor cells for tissue regeneration. If ES cells can be induced to differentiate using various methods to generate somatic stem cells or tissue-progenitor cells of the target organ, tissue regeneration can be performed efficiently and on a large scale by implanting these cells. However, there are problems that need to be resolved, including immunological rejection and ethical issues associated with the harvesting and use of ES cells. ES cells can be said to be the source of life and enable the creation of a complete human being. The current source of human ES cells is the surplus embryos that have become unnecessary after infertility treatment. The use of such embryos has been approved by considering this situation as a separate issue from abortion and questions such as at what point life begins. Thus, major issues remain such as the significance of debating the use of ES cells. Another major problem is immunological rejection that is associated with the use of ES cells harvested from surplus embryos. A method has been devised to generate ES cells from cloned embryos to address these issues [39]. Such cloned embryos are created by transfer of the recipient's somatic nucleus into the enucleated unfertilized egg, and ES cells are obtained from these cloned embryos. However, there is no guarantee that these ES cells are the same as ES cells obtained from a conventional method. That is, the ES cells might not be the same because the transplanted somatic nucleus might not be completely reprogrammed in the process of establishing the ES cell system from cloned embryos. Furthermore, there are still bioethical issues associated with the use of ES cells obtained from cloned embryos and such use is prohibited in many countries.

These problems can be avoided in the transplantation of mesenchymal stem cells directly harvested from autologous bone marrow. Thus, these mesenchymal stem

cells are promising, and various research studies are in progress on induction of differentiation into various tissues [40–42]. Bone marrow is the source of mesenchymal stem cells, and there are various cells in the harvested bone marrow. Mesenchymal stem cells need to be selected from these cells and transplanted after they are grown or after induction of differentiation. A problem that arises at this point involves how to select mesenchymal stem cells. The most common method is selection by cell surface antigen and involves cell sorting using a fluorescence-activated cell sorter (FACS). In this method, cells with fluorescently labeled antibodies are irradiated with a laser beam and the emitted fluorescence is detected. An oscillograph is used to show the distribution of cells by the difference in the intensity of fluorescence, and this method enables fractionation of cells. However, the most significant drawback of this method is that surface markers of mesenchymal stem cells have not yet been completely established. Although opinions differ by author, the established surface markers of mesenchymal cells are considered to be those previously mentioned [43–45]. Mesenchymal stem cells can be purified by long-term selective culture under certain conditions. However, this method is still in the experimental stage, and it is unknown whether it can be applied clinically.

Another problem has to do with the low number of mesenchymal stem cells. The proportion of mesenchymal stem cells among bone marrow stromal cells is said to be 1/10,000 in newborns and 1/400,000 in adults in their 50s, and 1/2,000,000 in adults in their 70s. Thus, the number of mesenchymal stem cells is very low in the bone marrow itself. Furthermore, mesenchymal stem cells undergo senescence, in which the cells no longer proliferate, after continued subculturing. Although ES cells have infinite division potential, senescence occurs in somatic stem cells which are downstream of ES cells [46]. As mentioned earlier, tissue regeneration using somatic stem cells can be performed by different methods. One method involves transplantation of stem cells as they are in the tissue or transplantation by systemic administration. Another method involves induction of differentiation of a certain cell lineage (targeting) and transplantation of these cells. In general, clinical application requires a large amount of progenitor cells of the specific target lineage. Thus, the aforementioned senescence becomes a problem.

When cells divide, they replicate chromosomal DNA. Normal DNA replicase cannot completely replicate the telomeric sites at both ends of the linear DNA genome. Therefore, the telomere length becomes shorter as cells repeatedly divide, and cell division ends when the telomere is no longer maintained. That is, cell proliferation ends at this point, and the number of cells does not increase thereafter. To maintain infinite division potential of cells, one can immortalize the cells by inserting the telomerase reverse transcriptase gene into the gene that controls this replication. Telomerase reverse transcriptase increases telomerase activities [47, 48]. When immortalized cells are generated in this way and transplanted, there is a risk of tumorigenesis, including cancer, in the long term. Furthermore, it has been reported that cells from long-term culture of mesenchymal stem cells themselves became cancerous [49]. Therefore, evaluation of safety for clinical application has become difficult.

iPS cells have been generated from autologous skin, muscles, and fat as research also advanced in ES cells and mesenchymal cells. The efficiency of iPS cell

generation is low and these cells are difficult to maintain. These problems are similar to those in ES cells. While the greatest disadvantages of ES cells are ethical issues and immunological rejection, iPS cells do not have these issues. However, iPS cells have problems such as malignant transformation in the process of differentiation and a low efficiency of iPS cell generation. More research needs to be done. ES cells, mesenchymal stem cells, and iPS cells have been discussed above, and each type of cell has advantages and disadvantages. It is important to use appropriate cells for a particular disease and regenerative environment.

1.6 Scaffold

In vivo, cells exist in an extracellular matrix called scaffold. The exceptions are cells that exist singly such as hematopoietic cells. That is, cells adhere to the extracellular matrix and maintain tissue and organ morphology. In many cases, when tissue is injured, a defect of its scaffold develops. Thus, it is necessary to provide a scaffold for regeneration to occur. An ideal scaffold has the following characteristics:

1. Has high tissue compatibility and does not cause immunological rejection as a foreign substance
2. Is porous to facilitate invasion of cells and supplying of oxygen and nutrients
3. Maintains its shape for a certain period of time, and prevents invasion of tissue (such as connective tissue) from the surroundings which would deprive the cells a place for regeneration
4. Degrades when tissue regeneration is complete

Current scaffolds are made of biopolymers, synthetic polymers, and inorganic compounds and include the following:

Biopolymeric materials: collagen, gelatin, glycosaminoglycan, and chitosan
Synthetic polymeric materials: polyglycolic acid, polylactic acid, poly(lactic-co-glycolic) acid, poly(lactic acid-co-ε-caprolactone), and poly-ε-caprolactone
Inorganic compounds: hydroxyapatite, tricalcium phosphate, and calcium carbonate

Among the aforementioned materials, collagen is a protein that is widely distributed in the body as an extracellular matrix. Collagen is a superior material as a scaffold for cell adhesion and growth and has wide-ranging applications. It has begun to be used clinically as artificial dermis [4], artificial tracheas [12], and artificial nerve conduits [13, 50]. Polyglycolic acid has been used as a protective membrane to prevent invasion of the surrounding tissue and to secure a space for regeneration. It has been applied clinically in artificial nerve conduits [13, 50] and guided tissue regeneration (GTR) membranes for periodontal tissue regeneration [51]. It has also been used in membranes for immunoisolation [52].

As mentioned earlier, a cell sheet developed by Okano et al. is composed of cells cultured on a scaffold of temperature-responsive polymers, indicating advancement in scaffold development [6, 7]. Temperature-responsive polymers are hydrophobic

at approximately 32°C and above and hydrophilic below 32°C. At a culture temperature (37 °C), the polymers have a hydrophobic surface onto which cells can adhere. The cells can be detached from the polymers after culturing when the temperature is decreased to room temperature (20–25°C). When these characteristics are used, cells cultured to confluence can be recovered as a cell sheet while maintaining the extracellular matrix. Conventionally, cells are adhered to and grown on the surface of the cultureware, and proteolytic enzymes (such as trypsin) are used to recover these cells. Thus, the enzymes cleave the cell-cell adhesion molecules and the cultured cells are recovered as individual cells. In addition, the enzymatic treatment weakens the cells themselves. In contrast, when cells are cultured using temperature-responsive polymers, enzymatic treatment is not used and intercellular proteins are not destroyed, enabling the recovery of cells as an intact sheet. Adhesion proteins are formed between the cells and the cultureware surface during culturing. Since these proteins are maintained and not destroyed, the recovered cell sheet can be promptly engrafted into a living tissue. In addition, multiple sheets can be stacked over one another so that the cell sheets can adhere to each other. Furthermore, the original functions of the cells are maintained because the cell sheets do not undergo enzymatic treatment.

Cell sheet engineering has been applied in the regeneration of the following tissues: corneal epithelial tissue using an oral mucosal cell sheet [53], myocardial tissue using a skeletal myoblast cell sheet [54], esophageal epithelial tissue using an oral mucosal cell sheet [55], periodontal tissue using a periodontal ligament cell sheet [56], and cartilage tissue. Thus, cell sheet engineering is a field with wide-ranging applications, enabling regeneration for local organ injuries.

Porous hydroxyapatite is a material that can be used as a scaffold for bone regeneration. It facilitates the induction and proliferation of cells such as osteoblasts, integrates with regenerated bone, and maintains a certain shape and strength. However, it is not bioabsorbable. Beta-tricalcium phosphate (b-TCP) is a bone substitute that is replaced by autologous bone after implantation. It is osteoinductive and osteoconductive and is absorbed by the body as bone regeneration proceeds. It has been effective in clinical application for bone regeneration in dentistry and orthopedic surgery [57, 58]. Instead of using hydroxyapatite as a bone substitute, some studies used the coral-shaped hydroxyapatite surface that had been coated with collagen for mucosal regeneration on its surface, and mastoid air cells were regenerated [59, 60].

1.7 Regulatory Factors

In tissue regeneration, regulatory factors and cytokines act to control cell growth and differentiation. Regulatory factors have various functions, including promotion as well as inhibition of cell growth and differentiation and promotion of apotosis. In the process of tissue regeneration, these factors do not act alone but multiple factors act simultaneously, and synergistically or antagonistically. Many

factors are called growth factors. However, the factors that act on hematopoietic cells are called cytokines, and the factors that act on the cells of the nervous system are called neutrophic factors. Many of these factors are not of one type but they often form families (of structurally related molecules that bind to a common receptor). Readers should refer to suitable publications for details on the functions of various factors. The following sections classify the factors by the receptors on which they act.

1.7.1 Growth Factors Binding to Tyrosine Kinase Receptors

Epidermal growth factors (EGFs), fibroblast growth factors (FBSs), hepatocyte growth factors (HGFs), and nerve growth factors (NGFs) bind to tyrosine kinase receptors. In many of these growth factors, signaling molecules with SH domains transmit the growth-promoting signal. These growth factors have major involvement in cell growth, differentiation, and migration as well as in organogenesis in early development.

Kanemaru et al. used a gelatin sponge soaked in a solution of basic fibroblast growth factor (b-FGF) to repair a tympanic membrane perforation [61]. A mechanical disruption was created at the edge of the tympanic membrane perforation, and the sponge was placed over the perforation. Fibrin glue was used as a sealant. This procedure enabled successful tympanic membrane regeneration. This treatment uses a unique method without cell transplantation. Namely, tissue stem cells/progenitor cells, which will be the source of tympanic membrane, are activated by mechanical stimulation at the margin of the tympanic membrane perforation [61]. Moreover, they also succeeded in regeneration of external auditory meatus by the same therapeutic strategy [62].

Hirano et al. administered b-FGF or HGF to atrophied vocal cords and successfully increased hyaluronic acid and decreased collagen of the vocal cord lamina propria. This method increased the vocal cord volume and viscoelasticity and improved vocalization [63].

1.7.2 Growth Factors Binding to Serine/Threonine Kinase Receptors

The factors that bind to this type of receptor include those of the transforming growth factor-beta (TGF-b) superfamily. The following factors of this superfamily are known other than TGF-b: bone morphogenetic protein (BMP), activin, and Müllerian-inhibiting factor (MIF). They bind to serine/threonine kinase receptors and transmit a signal through a group of proteins called “Smad.” In this process, Smad is phosphorylated and translocates into the nucleus to regulate transcription of the target gene and to transmit a signal. When the aforementioned HGF or EGF

acts on cells, tyrosine kinase receptors are activated, resulting in the inhibition of nuclear translocation of Smad proteins. Therefore, resistance to TGF-b and BMP occurs [64].

1.7.3 Cytokines Binding to Cytokine Receptors

The cytokine receptors include receptors for interleukins (such as IL-2, IL-3, IL-4, IL-5, and IL-6) and receptors for hematopoietic growth factors. These factors act on lymphocytes, hematopoietic cells, epithelial cells, and neurons. These cytokine receptors lack intrinsic tyrosine kinase activation. However, cytokines bind to the receptors to form complexes, enabling cytoplasmic tyrosine kinases to bind to the receptors for signal transduction.

1.7.4 Factors Binding to Other Receptors

The factors that bind to other receptors include Fas-ligand and tumor necrosis factor-alpha (TNF-a). These factors bind to Fas receptors and TNF receptors, respectively. Caspase activity occurs only after the receptors polymerize, and signal transduction for apoptosis occurs. Wnt signaling known from Drosophila studies also involves this type of factors [65, 66].

The major goals are to induce differentiation of stem cells in a specific direction using regulatory factors, to amplify the culture of the resulting cells, and to use these cells for tissue regeneration. Unfortunately, much is still unknown regarding which growth factors and cytokines are acting to what extent in the body, in what amounts, and at what time period. Studies have been conducted in a very pure environment (such as in serum-free medium) to examine the effects of various regulatory factors on stem cells and on induction of differentiation [67]. In any case, the elucidation of this signal network can be said to be an important key to tissue regeneration [67].

In general, the effects of various growth factors and cytokines are very short term. If one merely administers these regulatory factors from an external source, one can expect very minimal effects. Some type of technique is necessary to maintain these regulatory factors at certain concentrations for a certain period of time. One technique uses a drug delivery system (DDS) in which regulatory factors are incorporated into a bioabsorbable material and slowly released (sustained released) into the tissue targeted for regeneration [68]. Generally, in vivo, cell growth factors bind to the extracellular matrix through physical interaction, and subsequently the matrix is hydrolyzed in response to a signal from the body, resulting in the release of these factors. Tabata et al. created a system in which plasmid DNA and growth factors were incorporated into a gelatin hydrogel, a bioabsorbable polymer. These factors were released when the hydrogel degraded [68–70].

There have been efforts to develop treatments using the self-regulatory ability of a living body. They involve gene transfer via vectors to the target site for regeneration. These genes are associated with production of growth factors necessary for such regeneration. Treatments for Buerger disease, arteriosclerosis obliterans, and myocardial infarction are examples that use transfer of genes associated with the production of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [71–73].

1.8 Summary

It has been only 7 years since iPS cells that broke the preconceived notion of "completely differentiated somatic cells cannot be reprogrammed" were created Research in regenerative medicine has been advancing very rapidly. In contrast, the clinical application of these cells is proceeding slowly in regenerative therapy but is certainly advancing. We believe that regenerative medicine will be added to the existing treatments in the near future. In the otorhinolaryngological field and in the craniocervical regions, there are many organs in which morphological regeneration is closely associated with functional regeneration. In particular, when patients have facial or cervical deformity after head and neck cancer surgery, it would be wonderful for patients if lost tissue can be regenerated and natural morphology can be maintained. Since I have been involved in regenerative medicine, the thing that changed the most is the way I think about injured or impaired tissue. To get ideas on research, I began to think constantly about how wound healing occurs and what hinders natural regeneration. I hope that more researchers can participate and play an active role in regenerative therapy in the otorhinolaryngological field and in the head and neck region, which seems to be lagging behind other fields.

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Part II
Development and Regeneration of the Ear

Chapter 2
The Inner Ear

Juichi Ito

Abstract Diseases- and symptoms-related inner ear dysfunctions such as sensorineural hearing loss (SNHL), tinnitus, and vertigo are very difficult to treat. One of the main reasons is caused by the difficulty that inner ear sensory cells have little ability to regenerate after damage. There are more than 400,000 deaf or highly hearing-impaired people in Japan. One of 1,000 newborn babies is deaf. Therefore, recovering inner ear disorders has always been one of the most important priorities in the field of otolaryngology. In this chapter, the possible therapeutic strategies for inner ear regeneration are summarized. In early-phase cochlear damage, it is crucial to rescue auditory cells from cell death and promote self-repair cell activity. Induction of transdifferentiation is the next useful strategy. If no cell sources remain in the inner ear, cell transplantation then becomes the only choice to restore cell growth through regeneration. Using bionic materials is another remaining possible alternative approach. Detailed related inner ear regeneration is already described in another edition (Ito J. Regenerative medicine for the inner ear. Tokyo: Springer, 2014).

Keywords Inner ear • Regeneration • Stem cells • Transdifferentiation • Transplantation • Artificial auditory epithelium

2.1 Anatomy of the Inner Ear

Inner ear of mammals consists of the cochlea, the vestibule, and three semicircular canals. The lumen of the otic capsule is called bony labyrinth and the inner membranous wall is called membranous labyrinth. The space between the bony and membranous labyrinth is filled with perilymph. Inside the membranous labyrinth is called endolymphatic space, and is filled with endolymph. The perilymph is sodium rich and the endolymph is potassium rich.

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The sensory epithelium of the cochlea is the organ of Corti, in which sensory hair cells stay in array. The axis of the cochlea is known as the modiolus, in which the cochlear nerve runs. The cross section of the membranous labyrinth in the cochlea forms a triangular shape bordered by the basilar membrane, the stria vascularis, and the Reissner's membrane [1]. The endolymphatic space inside the cochlea is called scala media. The perilymphatic space facing the Reissner's membrane is called scala vestibuli, and the space facing the basilar membrane is called scala tympani. The basilar membrane in the basal turn is narrower and stiffer, leading to higher natural resonance frequency, while the apical turn leads to lower resonance. The organ of Corti is composed of hair cells and several types of supporting cells. When the sound vibration displaces the basilar membrane, the shearing force between the basilar and the tectorial membranes deflects the hair bundle at the apical surface of the inner hair cells and then is conveyed as the release of the neurotransmitter.

Approximately 3,500 inner hair cells exist in one human cochlea. The outer hair cells appear as three rows, and 12,000 outer hair cells exist in one human cochlea. The outer hair cells are innervated by type II afferent neurons with unmyelinated axons.

Loose connective tissue on the outer wall of the cochlear duct is the spiral ligament. The stria vascularis is a vascular-rich structure on the spiral ligament spreading from the attachment of Reissner's membrane to the spiral prominence. The stria vascularis functions to maintain the high potassium concentration and positive potential of the endolymph.

The vestibule composed of the utricle and saccule functions to sense linear acceleration. The space near the semicircular canals is called the utricle, while the other located near the cochlea is called the saccule. The sensory epithelium of the vestibule is located in the macula of the utricle and saccule. The utricular macula is located horizontally with a free margin. The saccular macula is attached to the medial wall of the saccule. Hair cells in the maculae are classified into type I and II hair cells, which are morphologically distinguishable, but the functional difference is not fully understood.

The three semicircular canals (lateral, superior, and posterior) contribute to the stimulus perception of three-dimensional head rotation. In each semicircular canal, the membranous labyrinth presents as a tube-like structure. At one end of each semicircular canal, the tube is dilated to form a spindle-like structure, which is known as the ampulla. A crescent-shaped structure (crista) is located inside the ampulla. The crista is attached to the outer wall of the ampulla, and the hair cells of the semicircular canals are located in the sensory epithelium of the crista.

2.2 Regeneration of the Inner Ear

2.2.1 Stem Cells

2.2.1.1 Inner Ear Stem Cells

Mammalian inner ears were reported to contain cells that had the ability to proliferate clonally and to differentiate into several types of inner ear cells. Regeneration was reported to occur in vestibules, while the mammalian

cochlear cells have been reported to have little ability to regenerate after damage. The inner ear has limited ability to regenerate, while there are other evidences indicating the existence of inner ear stem cells. Potential candidates of stem cells are supporting cells [2], tympanic border cells [3], and other candidates. The total number of them in the inner ear is estimated to be very small, and reported to decrease as the inner ear matures. Tympanic border cells identified as slow-cycling cells have the characteristics of stem cells [3]. Immunohistochemical analysis indicated that these cells stained positive for immature cell marker nestin. The number of slow-cycling cells in the tympanic border cells decreased dramatically in about 2 weeks after birth as the cochlea matured.

2.2.1.2 Embryonic Stem Cells (ESCs)

ESCs are widely used for the generation of knock-out/knock-in animals, chimeras, and clones. Nowadays, these genetically modified animals are indispensable tools for biological research and animal industry. Extending this study, ESCs were induced to differentiate into a wide variety of cell types including hepatocytes, pancreatic islet cells, blood cells, chondrocytes, cardiomyocytes, dopaminergic neurons, Schwann cells, retinal pigmented epithelium and so on, and expected to be applied in clinics.

2.2.1.3 Induced Pluripotent Stem Cells (iPSCs)

iPSCs are somatic-cell-derived stem cells similar to ESCs in terms of self-renewal and pluripotency [4]. iPSCs are similar to mouse ESCs in many points such as an expression of stem cell. The regeneration of the auditory pathway by transplants of fetal brain tissue was first demonstrated [5]. Since then several stem cell-based approaches to cure deafness using mesenchymal stem cells, neural stem cells, ESCs, and iPSCs have been adopted. Current major targets are cochlear hair cells and spiral ganglion neurons (SGNs), which are primarily compromised and unregenerable cells in sensorineural hearing loss. Inner ear hair cells of the mammals have very little capacity of regeneration [6]. This is one major reason for the difficulty in recovering from permanent profound hearing loss. One suggested modality for the recovery from the hearing impairment is the regeneration of hair cells. Hair cell induction from pluripotent stem cells has gradually become a reliable method. Hurdles still exist before the realization of hair cell regeneration. It is still necessary to improve the induction rate, and to develop the method to specifically induce cochlear hair cells. More fundamental difficulty is the in vivo application of induced hair cells. It is not realistic to transplant mature hair cells and to arrange them in the cochlear epithelium [7]. Introduction of stem cells that are programmed to differentiate to inner ear sensory fates and introduction of stem cell-based factor releasing cells may also be other possible strategies.

2.2.2 Transdifferentiation

Transdifferentiation is defined as the changing of one terminally differentiated cell type into another without producing a pluripotent intermediate. In the regeneration of avian auditory sensory epithelia, supporting cells that are located around hair cells spontaneously transdifferentiate into hair cells after hair cell injury. Although postnatal mammalian hair cells never regenerate spontaneously, Atoh1 overexpression or the inhibition of Notch signaling have been reported to induce regeneration of hair cells through transdifferentiation of supporting cells in rodents. In birds, vestibular hair cells regenerate throughout their life [8] and even auditory hair cells can regenerate after injury [9]. Two underlying mechanisms of these regenerative processes have been identified. One of the mechanisms is the direct transdifferentiation of supporting cells into hair cells [10]. Transdifferentiation may occur readily between hair and supporting cells in birds because both cells share common progenitors during their development. Among several transcription factors related to the development of hair cells, Atoh1 is the most promising factor for the regeneration of hair cells. Atoh1 was overexpressed in postnatal mammalian cochlea in vitro by using electroporation [11] and in vivo by using adenovirus vectors [12], to induce transdifferentiation of hair cells. Transdifferentiation from supporting cells to hair cells was examined by inhibiting Notch signaling. Similar results were obtained through the pharmacological inhibition of Notch signaling by using a gamma-secretase inhibitor [13]. Gamma-secretase inhibitor treatment on adult cochlea with injured hair cells caused the transdifferentiation of hair cells from supporting cells and functional recovery, although the hearing threshold change was very small [14].

2.2.3 Cell Transplantation

Cell transplantation into the cochlea would be an alternative strategy when there is not a stem cell population enough to regenerate damaged hair cells. Stem cells described before are prime candidates for cell-based therapies since they potentially can replace damaged cells when administered into a target organ. Several trials have investigated the effect of stem cell therapy in the inner ear using exogenous stem cells. Review history of experimental cell transplantation into the cochlea beginning with the pioneering report [15]. In this study, neural stem cells derived from adult rat hippocampus were transplanted to the cochlea of neonatal rat. As recipient grows as old as 4 weeks, histological analysis demonstrated that a part of transplanted cells had survived in the cochlea. Moreover, some of the transplanted stem cells migrated into the organ of Corti and showed hair cell-like morphology. The observation suggests that transplantation of exogenous cells to the cochlea could be an alternative strategy for treatment of the damaged cochlea and sensorineural hearing loss. Survival and differentiation of neural stem cells in the adult mouse cochlea damaged by injection of neomycin was also reported [16]. After transplantation, immunohistochemistry revealed that fewer parts of transplanted neural stem cells were

found in the perilymphatic space and differentiated into hair cell-like cells in the vestibular sensory epithelium, but not in the cochlea. Neural stem cells were transplanted in the mouse inner ear through horizontal semicircular canal and showed histological results indicating that transplanted neural stem cells express neurotrophic factor, such as BDNF and GDNF after transplantation [17]. These data suggest that transplantation of exogenous stem cells does not always lead to differentiation of transplanted cells to hair cells, but induces protective effects on hair cells and/or surrounding supporting cells against insults in the cochlea. Mouse embryonic stem cells were transplanted and expressing GFP into the adult mouse inner ear damaged by neomycin prior to cell transplantation, while none of the transplanted cells were observed as hair cell phenotype [18]. Fetal rat otocyst cells on exogenous cell were transplanted into the rat inner ear damaged by acoustic overstimulation [19]. After transplantation, a few grafted fetal otic epithelial cells were integrated in the supporting cell layer of the damaged cochlea in host animals, whereas most of transplants were found in the perilymphatic space or attaching to the cochlear lateral wall.

Another target of cell transplantation is the spiral ganglion neurons (SGNs). SGNs conduct auditory information from the inner hair cells to the cochlear nucleus. Most patients with a loss of SGNs exhibit severe degeneration of the hair cells. In the clinical setting, therefore, the drawback of an SGN deficit is a poor outcome after cochlear implantation. For successful reconstruction of auditory neurons, it would be logical to guide transplanted cells to recapitulate the embryological developmental process [20]. The more undifferentiated cells we choose as donor cells including ES cells and iPS cells [21, 22], the more advanced techniques to control cell differentiation and cell fate should be sought.

2.2.4 Newly Invented Bionic Material

A new hearing device that takes the place of cochlear implantation has recently been developed. The aim of this project is to develop as novel therapeutic method for sensorineural hearing loss (SNHL) using a newly invented artificial sensory organ. The development of a new and totally implantable device is accomplished by an incorporation of nanotechnology, cochlear micromechanics, and tissue engineering together with regenerative medicine for the inner ear. The new device is named artificial sensory epithelium (AAE), since this device imitates functions of auditory sensory epithelia [23]. The AAE implantation involves: (1) surgically exposing the bony wall of the scala tympani to insert into the cochlea and placing it on the surface of the basilar membrane; (2) when a sound reaches the cochlea, the basilar membrane vibrates; (3) thus vibrating the AAE placed on the basilar membrane; (4) AAE distortions caused by vibrations then generate electricity via piezoelectric effect (an effect which converts mechanical movements into electric signals) to stimulate the auditory nerve; and (5) acoustic input is next transmitted to the central nervous system. AAE, as its name implies, imitates the functions of sensory epithelia. This

means that elements required for AAE transform vibratory movements into electric signals with frequency characteristics. To confirm that these required elements can be accomplished by AAE, a prototype of AAE was fabricated to verify the validity of the basic mechanisms. This prototype of AAE has a gradient in its width, which is also a characteristic of the basilar membrane. The output voltages of the piezoelectric device are considered to be enough to stimulating neurons. AAE project is thought to be a mechanical regeneration.

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Chapter 3
Regeneration of the Soft Tissue Defects of the External Auditory Meatus

Shin-ichi Kanemaru

Abstract The aim of this study is to establish a regenerative treatment for soft tissue defects of the external auditory meatus (EAM) without conventional surgical therapy. Thirty patients with new or old EAM defects without active inflammation were randomly selected. Ages ranged from 15 to 82 (average age of 55). Gelatin sponge, basic fibroblast growth factor (b-FGF), fibrin glue, and waterproof transparent dressing were used in the repair procedure. Patients were divided into two groups: treatment with ($n = 20$) and without ($n = 10$) b-FGF. After mechanically disrupting the edge of the EAM defect, gelatin sponge immersed in b-FGF was placed over the defect and covered with fibrin glue. In cases of extensive EAM defects, the EAM was filled with gelatin sponge/b-FGF, and the auricle was wrapped in waterproof dressing. Three weeks post-procedure, crust over the defect was removed. If complete defect closure was not achieved after one treatment course, the treatment was repeated until three times. Evaluation of complete closure of EAM defects was performed 3 months posttreatment. Complete closure of the EAM defect was achieved within three treatment courses in 90 % (18/20) and 20 % (2/10) of the patients with or without b-FGF, respectively. No inflammation/infection or severe sequelae were observed.

This study demonstrated the effectiveness of combining gelatin sponge, b-FGF, and fibrin glue for EAM defect regeneration. This innovative regenerative therapy is an easy, simple, cost-effective, and minimally invasive method for treating EAM defects.

Keywords Regeneration • External auditory meatus defects • Gelatin sponge • Basic fibroblast growth factor • Fibrin glue • In situ tissue engineering

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3.1 Introduction

The external auditory meatus (EAM) is divided into cartilaginous portion in its outer 1/3 and bony portion in its inner 2/3. The surface of EAM is lined by keratinizing stratified squamous epithelium. This epithelium is in continuity with the lateral surface of the tympanic membrane (Fig. 3.1). As there is little subcutaneous tissue, it is very difficult to cure the defect spontaneously, once it reached the bone.

Bone exposed defect (BD) of the EAM can result from various causes including injury, disease, congenital anomalies, and postoperative damage. They are a frequent cause of recurrent infection often accompanied by pain, malodor, otorrhea, and bleeding. This condition imposes many restrictions on the patient's daily life such as the inability to take a bath, shampoo one's hair, or go swimming. BD of the EAM is a particularly serious problem for patients who need to wear hearing aids.

To date, almost all treatments for these types of defects have involved surgical repair requiring incisions of the postauricular skin, the harvesting of autologous tissue, and other invasive procedures. These surgical treatments demand that patients endure multiple discomforts including anesthesia, bed rest, and/or hospitalization, the high-priced cost of the operation, restrictions on daily life, and cosmetic sequelae to name a few. More importantly, these types of invasive treatments are not always successful and often pose other risks including the development of iatrogenic cholesteatomas and deformity of the EAM. Additionally, conventional surgical techniques are unable to restore the normal shape of the EAM. The undamaged EAM has a self-cleaning function, which keeps the sound pathway clear of debris. A mechanism of epithelial migration moves surface keratin laterally from the tympanic membrane toward the ear canal opening. This desquamation and skin migration aids in moving objects out of the ear canal. Therefore, in order to maintain normal EAM morphology, it is very important that the EAM retain these functions.

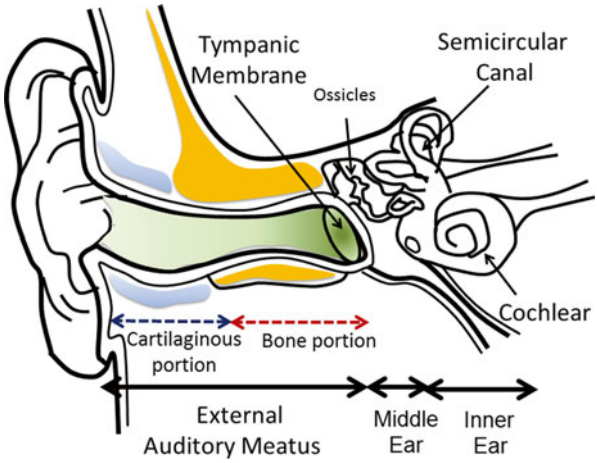

Fig. 3.1 Anatomy of ear

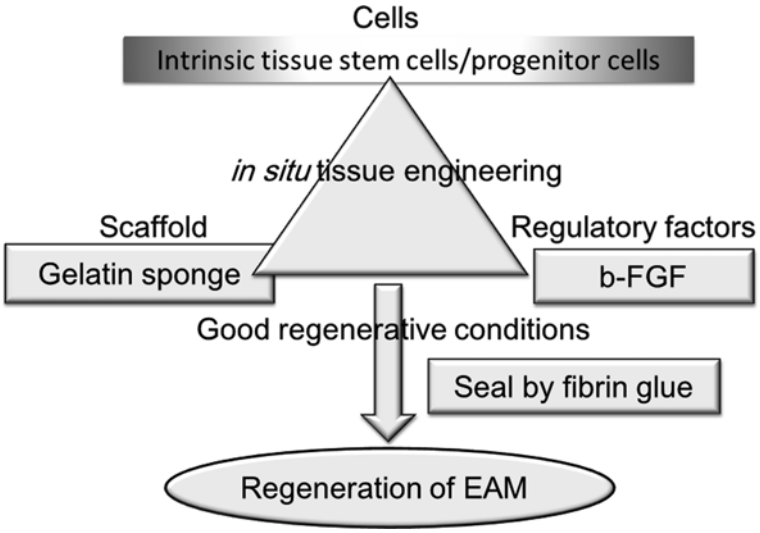

Fig. 3.2 Schematic of the tissue engineering triad and our strategy for regenerating BDs of the EAM. According to the principles of *in situ* tissue engineering, regeneration of tissues/organs requires the introduction of three basic elements – cells, scaffolds, and regulatory factors – under suitable conditions. In this clinical study, we introduced just two of these elements – scaffolding and a regulatory factor – as cells were expected to be endogenously supplied from the mechanically disrupted edge of the defect of EAC. That is, mechanical disruption of the defect edge may stimulate the activity of tissue stem cells/progenitor cells of the EAC in this region

The need for an effective treatment strategy is not limited to large BD of the EAM, as even acute BD often fails to heal spontaneously. This is due to the fact that the soft tissue of the EAM is very thin and somewhat limited in blood supply. Thus, it is very important that regeneration strategies for BD of the EAM consist of a better arrangement of regenerative conditions such as ensuring an abundant blood supply as well as an efficient seal for the prevention of infection and the maintenance of moisture within the tissue.

As present treatments for BD of the EAM stand on the concept of reconstructive surgery, they are woefully insufficient from the points of both morphology and function.

We newly developed the regenerative treatment for BD of the EAM as follows (Fig. 3.2).

3.2 Patients and Therapeutic Materials

Thirty patients consisting of 12 males and 18 females participated in this clinical study. These patients were selected from outpatients with EAM cholesteatomas ($n=13$), EAM cholesteatomas following middle ear surgery ($n=4$), traumatic BDs of the EAM ($n=6$), BDs of the EAM following middle ear surgery ($n=3$), and benign tumor of the EAM ($n=4$). Patient ages ranged from 15 to 82, with an average age of 55.

Fig. 3.3 (**a-1**) b-FGF. (**a-2**) Gelatin sponge. (**b-1**) Trimmed gelatin sponge. (**b-2**) Gelatin sponge with b-FGF

They were classified into three grades based on the size of their BDs: below 100 mm^2 as Grade I ($n=11$), from 100 to 200 mm^2 as Grade II ($n=12$), and over 200 mm^2 as Grade III ($n=7$). Patients were divided into two groups: the b-FGF group ($n=20$: Grade I=7, II=8 and III=5) and the control group ($n=10$: Grade I=4, II=4, and III=2).

Materials used for repairing the BD included a gelatin sponge (Spongel: Astellas Pharma Inc. Tokyo, Japan) with basic fibroblast growth factor (b-FGF, Fibrast: Kaken Pharma Co., Ltd Tokyo, Japan) and a fibrin glue (Bolheal: Kaketsuken, Kumamoto, Japan or Beriplast: CSL Behring K.K., Tokyo, Japan) (Fig. 3.3a, b).

The study subjects were limited to patients who fully understood the procedure and agreed to pursue this new treatment by signing "Informed Consent" documents. This new therapy was approved by the ethical committee of the Medical Research

Institute Kitano Hospital in Osaka and Kanai Hospital in Kyoto Japan; all procedures followed were in accordance with the Helsinki Declaration.

3.3 Therapeutic Procedures

Figure 3.4 shows all procedures. The soft tissue of the EAM was fully anesthetized by applying a cotton ball soaked in 4 % lidocaine for 15 min in most cases of Grade I or II. The patients of Grade III and a part of Grade I and II were anesthetized by injecting 1.0 % lidocaine with epinephrine. In cases with cholesteatomas or benign tumors, the entire growth was removed along with the surrounding tissue. In cases of simple soft tissue defects, a mechanical disruption of the defect edge was created. Both of the procedures described above were performed under the microscope by oto-microsurgery. In the b-FGF group, a gelatin sponge slightly larger than the defect was immersed in b-FGF (10–300 μg: 100 μg/mL of Trafermin, a recombinant human b-FGF); the sponge was placed such that it was in contact with the edge of the defect. The bone surface had been made smooth using a micro-osteotrite in the case of severe cholesteatoma. In the control group, the same treatment was performed using a gelatin sponge that had been immersed in saline. Fibrin glue was then dripped over the sponge to form a seal. In cases with very large BDs comprising the entire area of the EAM, the EAM was packed with gelatin sponge soaked in b-FGF and sealed with fibrin glue. In these extreme cases, the auricle was then dressed with a waterproof transparent dressing (Tegaderm: Sumitomo 3M Limited, Tokyo, Japan) (Fig. 3.4).

Following the procedure, patients were advised to avoid touching their ear and to keep their ear dry and were ordered to revisit the hospital 2 or 3 weeks after treatment at which point any crust over the defect was removed.

3.4 Evaluation

Three weeks after the procedure, the effectiveness of this treatment was evaluated by the images of fiberscope and microscope. In cases in which complete closure of the defect was not achieved, the above treatment was repeated up to three times. The final evaluation was performed 3 months after the initial treatment. The success rate was determined based on the rate of complete closure of the EAM BDs within three courses of treatment.

The occurrence of negative side effects and sequelae were also evaluated. Average follow-up period was 24 months (from 3 to 30 months).

In five cases, in order to evaluate a self-cleaning function, which keeps regenerated EAM clear of debris, we observed migration of pigment (Pyoctanin/Crystal violet: Methylrosanilinium Chloride) marked on the regenerated site for 4 weeks (Fig. 3.8).

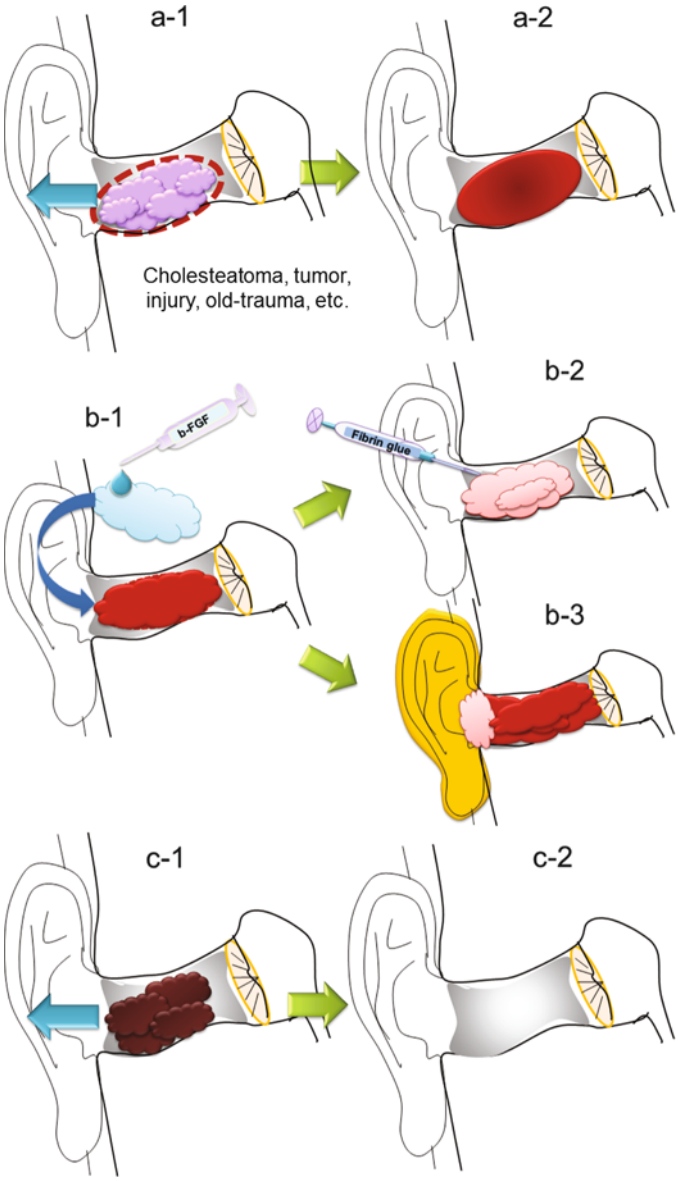

Fig. 3.4 Procedures used in this treatment: (**a-1**) After fully anesthetizing the EAM by application of a cotton ball soaked in 4 % lidocaine for 15 min, any lesions such as cholesteatomas or benign tumors are resected along the dotted line. (**a-2**) A mechanical disruption of the EAM defect edge is created. Depending on the cases, a concave of the bone is made smooth by a drill/a micro-sharp spoon after removing the matrix of cholesteatoma or a tumor. (**b-1**) Trimmed gelatin sponge soaked in b-FGF is placed over the defect. (**b-2**) The gelatin sponge is sealed with fibrin glue. (**b-3**) In cases with very large BDs comprising the entire area of the EAM, after the EAM was packed with gelatin sponge soaked in b-FGF and sealed with fibrin glue, the auricle was then dressed with a waterproof transparent dressing. (**c-1**) Three weeks after treatment, crust over the EAM defect is removed. (**c-2**) In case complete regeneration has not been achieved, the above same treatment is performed repeatedly until three times

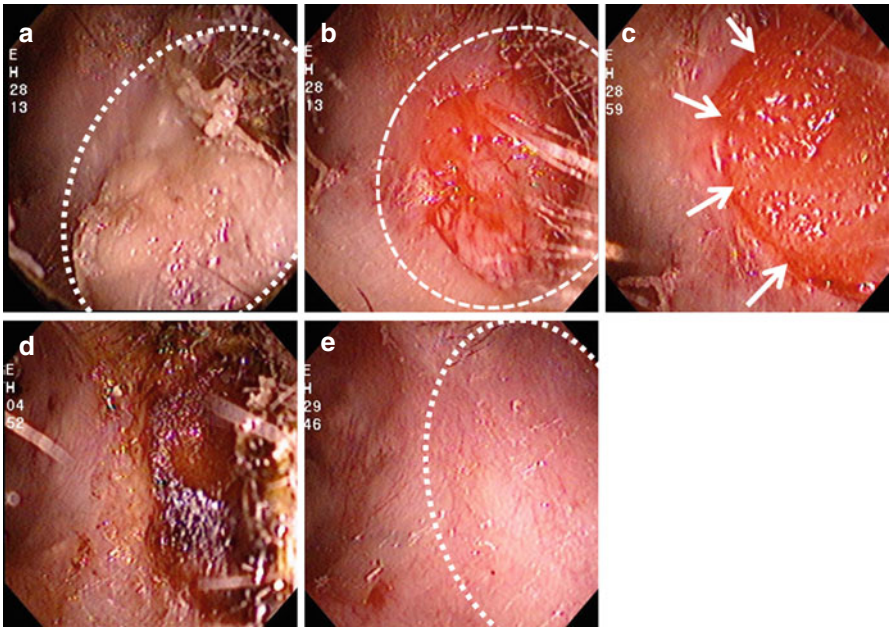

Fig. 3.5 Typical case of BD regeneration in a 67-year-old female with EAM cholesteatoma after middle ear surgery (Grade II). (**a**) White dotted circle indicates the cholesteatoma. (**b**) After the cholesteatoma was removed, a portion of the bone was exposed (*white dotted circle*). (**c**) Gelatin sponge containing b-FGF was placed into/over the defect and sealed with fibrin glue (*white arrows*). (**d**) Three weeks after treatment, dried gelatin sponge and crust covered the defect. (**e**) One month after treatment, the BD of the EAM was completely regenerated (*white dotted circle*)

3.5 Results

3.5.1 Complete BD Closure Rates

Figures 3.5, 3.6, 3.7, and 3.8 illustrate typical cases of regenerated BDs.

The overall rates of complete closure of the BDs were 90 % (18/20) in the b-FGF group and 20 % (2/10) in the control group. There were significant differences in the complete closure rates within each grade between the two groups. In the b-FGF group, the number/rate of the cases in which the BDs were able to close completely after one treatment, two treatments, or three treatments was 13 (65 %), 3 (15 %), and 2 (10 %), respectively. In two cases (10 %), the defects had not closed completely by the end of the third treatment. One of these two were postoperative cases (Grade II), the result of middle ear surgery more than 10 years before this regenerative treatment; in the other case of incomplete closure, the EAM defect was the result of thermal injury (Grade III).

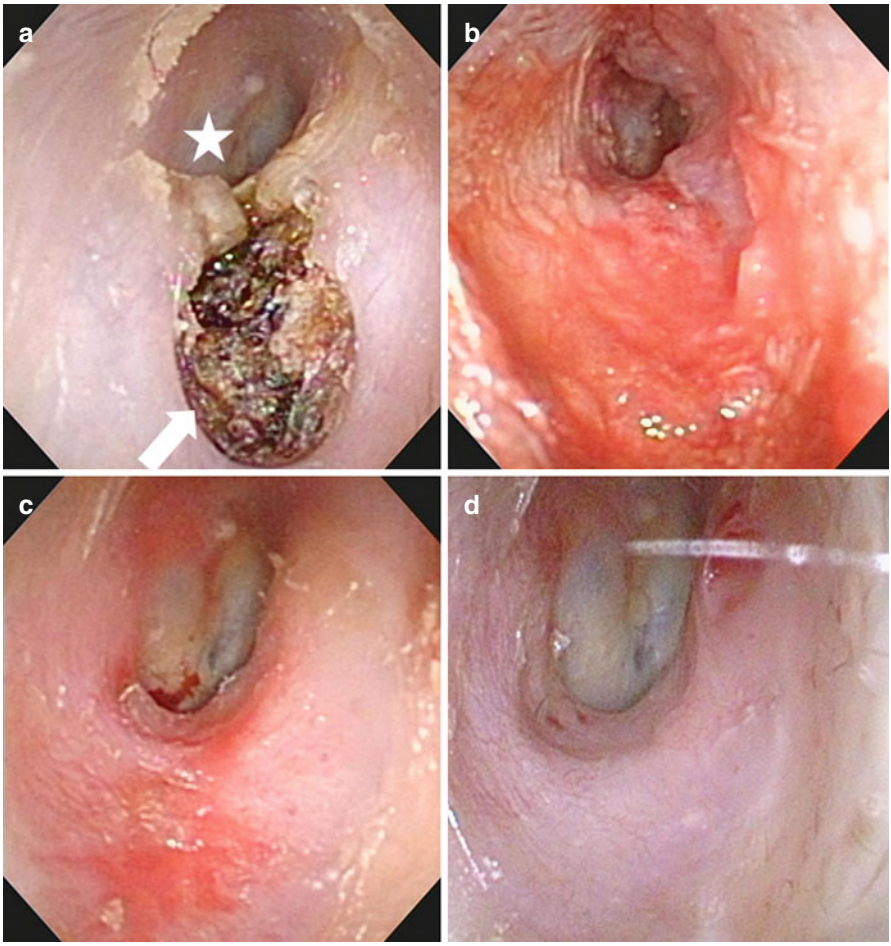

Fig. 3.6 Example of cholesteatoma with extreme bone defect of the EAM in a 71-year-old male. (**a**) Before treatment. *White asterisk* tympanic membrane, *white arrow* cholesteatoma with extreme bone defect of the EAM. (**b**) One month after treatment. (**c**) Two months after treatment. (**d**) One year after treatment, the BD of the EAM was completely regenerated

3.5.2 BD Size and Number of Required Treatments

Table 3.1 shows that the majority of cases in the b-FGF group required one or two treatments for complete regeneration of the defect.

3.5.3 Self-Cleaning Function

In all five cases of which EAM including the regenerated site was marked on by pigment (Pyoctanin), migration of pigment toward the orifice of EAM was observed as time advanced. This indicates that regenerated EAMs keep the normal self-cleaning function for clear of debris (Fig. 3.9).

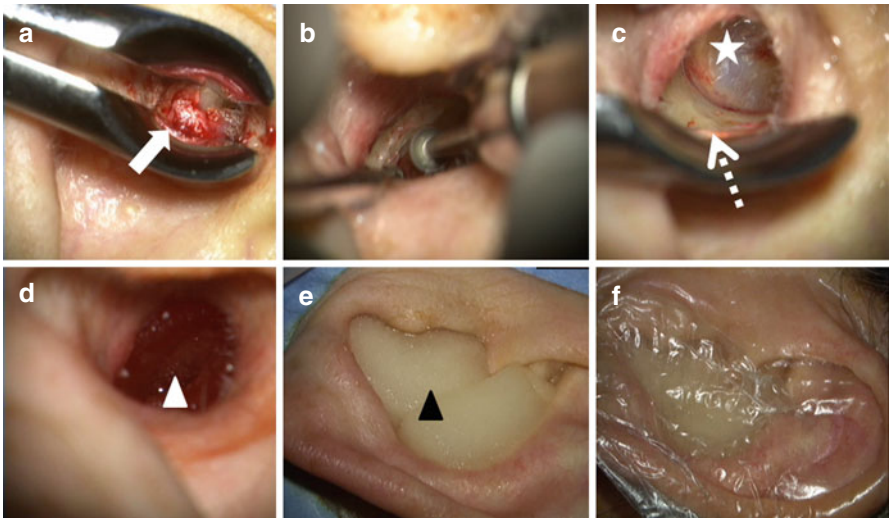

Fig. 3.7 Example of cholesteatoma with a very large BD encompassing the entire area of the EAM in a 72-year-old female. (**a**) *White arrow* indicates the cholesteatoma in the left EAM. (**b**) After resection of cholesteatoma, the bone surface of EAM is making smooth by diamond bar. (**c**) *White dotted arrow* indicates the BD of the EAM after resection of the cholesteatoma. *White asterisk* indicates the tympanic membrane. (**d**) Gelatin sponge with b-FGF (*white arrow head*) is placed into/over the defect across the entire EAM. (**e**) The orifice of the EAM is packed with gelatin sponge soaked with b-FGF (*black arrow head*). (**f**) The auricle is covered with a transparent waterproof dressing

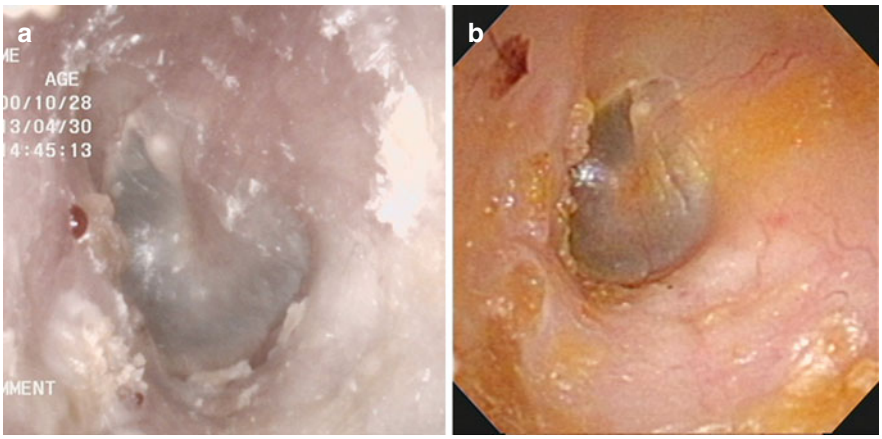

Fig. 3.8 These figures show the EAM before and after treatment in the case of Fig. 3.7. (**a**) Before treatment. (**b**) One month after treatment. (**c**) Three months after treatment. (**d**) Six months after treatment

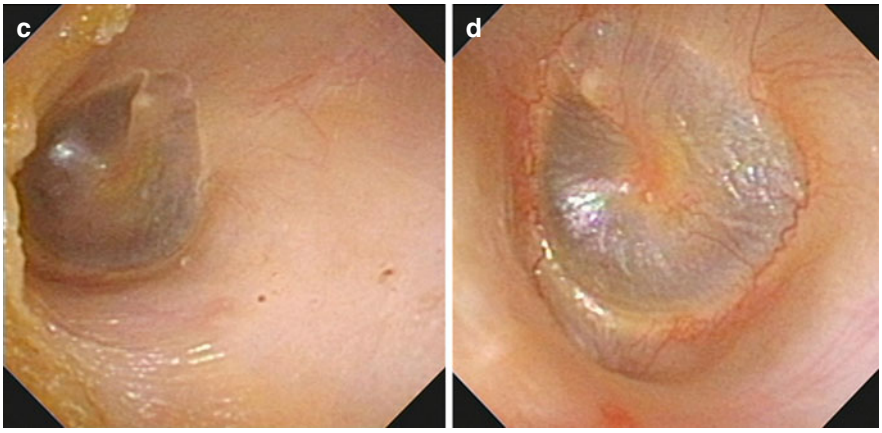

Fig. 3.8 (continued)

Table 3.1 Summarized results of the b-FGF group

Grade[a] ($n=20$)	I ($n=7$)	II ($n=8$)	III ($n=5$)
Number of treatments[b] (average)	1–2 (1.1)	1–2 (1.3)	1–3 (2.0)
Rate of complete closure of the soft tissue defects	100 % (7/7)	87.5 % (7/8)	80.0 % (4/5)
Average procedure time (minutes)	12	17	35
Disadvantages/sequelae	Nothing	Nothing	Recurrent cholesteatoma $n=1$

[a]Size of the EAM soft tissue defects: Grade I $\leq$100 mm^2, Grade II 100–200 mm^2, Grade III >200 mm^2
[b]In only succeeded cases

3.5.4 Complications

In one case of EAM cholesteatoma in the b-FGF group, a recurrent small cholesteatoma was observed 3 months after treatment. This was subsequently removed under a microscope. Though no infection or severe sequelae were observed in any patient, the patients whose auricles were dressed with a waterproof transparent dressing complained of itching of their EACs.

3.6 Discussion

Current standard treatments for BDs of the EAM are methods to foster epithelialization by skin grafts, fascia grafts, and artificial tissue matrix [1–3]. It is often necessary for them except an artificial material to harvest of autologous tissue and to

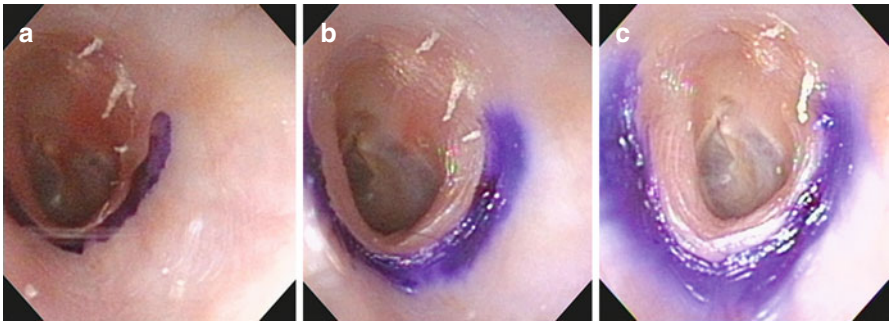

Fig. 3.9 Evaluation of a self-cleaning function of the regenerated EAM. (**a**) Six months after the treatment, EAM including the regenerated site was marked on by pigment (Pyoctanin). (**b**) Two weeks after marking, pigment move toward the orifice of EAM. (**c**) Four weeks after marking, pigment move toward the orifice of EAM farther

make a postauricular skin incision. The bigger BDs of the EAM are, the more difficult they are repaired. Repaired BDs are not always normal ones and stenosis/deformity of EAM is often observed. Though this tissue engineering treatment does not compare outcomes with current standard of care methods, there are lots of advantages in this treatment. It is noteworthy that normal shape of EAM can be regenerated by this treatment without external skin incisions and harvesting of autologous tissues. Moreover, though it depends on the cases, time to heal of this treatment is much shorter than that of pervious treatments [1–3].

The main principles of tissue engineering and regenerative medicine teach us to consider not only the three basic elements required for regeneration – cells, scaffolds, and regulatory factors – but also the ideal environmental conditions under which regeneration must occur [4–8].

The proper selection and combination of these three basic elements is essential for regeneration. In this clinical study, however, we introduced just two of these elements – scaffolding and a regulatory factor – as cells were expected to be endogenously supplied from the mechanically disrupted edge of the defect [6–8] (Fig. 3.2). This is another advantage of this novel therapy, as the need for cell transplantation would make it difficult to widely apply this therapy as an outpatient procedure. The clinical application of stem cell transplantation in regenerative therapies would require the establishment of a large regenerative medicine community and the development of personalized treatment solutions. However, the potential exists for endogenous tissue stem cells and/or progenitor cells to play an important role in regeneration.

In this study, there were two cases in which the defect had not closed completely by the end of the third treatment; they were cases of either postoperative or thermal injury. Similar results were observed in our previous study of tympanic membrane regeneration [9]. These results suggest that it is more difficult for cases of postoperative or thermal injury to regenerate compared with cases of simple defects. There are two possibilities for this observation: One is that normal anatomical structures including endogenous stem cells and/or progenitor cells are destroyed by previous

operations or thermal injury. Another possibility is that cicatricial tissue formed after the previous operation or thermal injury prevents new cells produced by the stem cells/progenitor cells from migrating to the region of the defect. In either case, the endogenous stem/progenitor cells are impeded from aiding in the regeneration of the soft tissue of the EAM.

In this clinical outcome, there is a tendency that complete BD closure rates turn down as increasing the number of required treatments. Possible reasons for it are as follows; becoming worse for regenerative conditions and/or exhaustion of stem/progenitor cells by repeating treatments. Interestingly, this tendency was also observed in our previous study: regeneration of tympanic membrane [9].

We selected the combination of a gelatin sponge as a scaffold and b-FGF as a regulatory factor (Figs. 3.2 and 3.3) as several reports have demonstrated that the use of growth factors and repair materials have the ability to improve tissue repair in other animals [10–13]. However, the precise combination of factors and materials for regenerating BDs of the EAM are yet to be determined.

There are few growth factors currently available to clinical applications. Basic-FGF is one of these clinically available growth factors and is typically used for treatment of skin ulcers and decubitus because it has strong inductive effects on both fibroblasts (from which the name comes) and blood capillaries [12, 13]. This former property is particularly suitable for regeneration of EAM soft tissue because the largest part of the EAM consists of two layers, the lower layer being comprised of fibrous tissue produced by fibroblasts. The capillary-inducing ability of b-FGF is also beneficial as it may foster improvements in the local regenerative environment.

One disadvantage to using a growth factor such as b-FGF is the possibility that it may accelerate the growth of cholesteatomas and tumors [14]. Specifically, it has been reported that cholesteatomas express the receptor for b-FGF. Therefore, if a part of cholesteatoma remains, b-FGF accelerates growth of residual cholesteatoma. After perfect removal of cholesteatoma, however, b-FGF does not cause iatrogenic cholesteatoma. Results of this study and our previous report support it [9].

We selected gelatin sponge as our scaffold material of choice. Gelatin sponge is made of a protein extracted from the collagen found inside the connective tissues of animals and is often used for its hemostatic properties in medical procedures [15]. Gelatin sponge has a high affinity for living tissues and is absorbable in vivo within a few months. Another benefit of gelatin sponge is that it can fit into BDs of any size or shape and its gel form makes it easy to handle. Observations of the regenerative processes as it was occurring in the EAM revealed that cells will grow into a gelatin sponge and grow to occupy a large space. Therefore, after providing a large surface area for cell growth, most portions of the gelatin sponge are extruded outside and the gelatin sponge remained of the regenerating region [16]. A gelatin sponge works not only as a scaffold for the regeneration of BDs but also as a sustained release substrate for b-FGF [17, 18]. These characteristics of gelatin sponge make it particularly advantageous for use in regenerating BDs of the EAM.

As a final consideration, it is also very important to optimize the regenerative conditions [7, 15, 16]. In the last step of our procedure, we seal the gelatin sponge

Table 3.2 Remarkable advantages of this new therapy

1. No external skin incisions or harvesting of autologous tissues is required.
2. It can be widely applied for use in BDs of various sizes/shapes.
3. This technique is a simple/easy outpatient procedure that can be completed within about 30 min.
4. No restrictions are placed on the patient's daily life.
5. No severe sequelae or known complications are associated with this procedure.
6. It has high success/cure rates.
7. It is possible to fully regenerate a normal EAM.
8. It is very cost-effective and safe.

with a fibrin glue to insulate it from the outside creating optimal regenerative conditions. Not only does fibrin glue help to keep the sponge in place, it also prevents cells from drying out and provides a barrier to infection, thus promoting ideal culture conditions in vivo [9].

In this clinical study, we establish a new regenerative therapy for repairing BDs of the EAM without the need for conventional surgeries. This tissue engineering strategy may also be applicable to other therapeutic targets.

Moreover, this regenerative therapy reduces the patient's psychological, physical, and economic burden. The benefits of this new therapy to a large amount of patients will be immeasurable. Table 3.2 shows remarkable advantages of this new therapy.

3.7 Conclusions

1. We developed a new regenerative treatment for BDs of the EAM without the need for conventional surgical procedures by using a combination of b-FGF, gelatin sponge, and fibrin glue.
2. In this new tissue-engineering therapy, we achieved complete regeneration of normal EAM with a success rate greater than 90 %.
3. This therapy makes it possible to regenerate not only morphology of EAM but also physiology of EAM.
4. This is an innovative therapy with the benefits of being an easy, simple, safe, cost-effective, and minimally invasive outpatient procedure.

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Chapter 4
Regeneration of the Tympanic Membrane

Shin-ichi Kanemaru

Abstract The objective of this study is to establish a tissue-engineering therapy for the treatment of large tympanic membrane perforation (TMP) without the need for conventional surgical therapy; 63 chronic TMPs were randomly selected from outpatients; 53 chronic TMPs were randomly assigned to the b-FGF group; and the remaining 10 chronic TMPs to the control group. Materials used for the TM repair were gelatin sponge and fibrin glue with/without b-FGF. After creating a mechanical disruption of the edge of the TMP, a gelatin sponge was immersed in b-FGF or saline (for the control group) and placed over the perforation. Fibrin glue was dripped over the sponge as a sealant. The effectiveness of this therapy was evaluated by closure rates, hearing level and sequelae 3 weeks after treatment. The treatment was repeated up to 4 times for cases in which complete closure of the TMP was not achieved after one round of treatment.

Complete closure of the TMP was achieved in over 98.1 % (52/53) of the patients in the b-FGF group and 10 % (1/10) of the patients in the control group. The average hearing level of all patients with successful TM repair was improved. Serious sequelae were not observed in any patient.

This study demonstrates that a combination of gelatin sponge, b-FGF, and fibrin glue enables the regeneration of the TM without conventional operative procedures. This innovative regenerative therapy is an easy, safe, cost-effective, and minimally invasive outpatient treatment.

Keywords Tympanic membrane perforation • Tissue-engineering gelatin sponge • Basic fibroblast growth factor (b-FGF) • Fibrin glue

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4.1 Introduction

Patients with chronic tympanic membrane perforation (TMP) have disadvantages including hearing loss, reduction of discrimination ability, and the indispositions of tinnitus and aural fullness. In addition to these problems, these patients must also endure many restrictions on their daily life due to the high risk of infection.

No treatment for large or total TMP except for surgical procedures such as tympanoplasty or myringoplasty is available at present. These surgical treatments, however, are accompanied by many drawbacks such as skin incisions, harvesting of autologous tissue, sequelae, potential failure of the operation, and high financial cost.

While chronic and large TMPs fail to heal spontaneously, almost all acute and small TMPs, such as those caused by injury, will naturally heal themselves. Considering this innate potential for repair, there is a possibility that regeneration may be possible for chronic and large TMP if adequate conditions for growth are made available.

In this study, applying the principles of modern tissue engineering, we developed a novel therapy for large/total TMP without the need for conventional surgical treatment stands on the concept of reconstructive medicine [1].

4.2 Patients and Therapeutic Materials

In this study, 56 patients with 63 chronic TMPs were randomly selected from a group of outpatients; 48 patients with 53 chronic TMPs were randomly assigned to the b-FGF group; and the remaining 8 patients with 10 chronic TMPs were assigned to the control group. Inclusion in the study was limited to patients who could fully understand the procedure and agreed to pursue this new treatment by signing "Informed Consent" documents. This new therapy was approved by the ethical committee of Kanai Hospital in Kyoto Japan and the procedures followed were in accordance with the Helsinki Declaration.

The patients' profiles are shown in Table 4.1.

Table 4.1 The patients' profiles

Patients/ears: $n=56/63$ (Male: 26, Female: 30), Age: 10–85 (Avg. 55)			
	Total ears $n=63$	b-FGF group 53	Control group 10
Otitis media with TMP without inflammation	37	32	5
Postoperatively reperforated TM	6	6	0
Old traumatic TMP	6	5	1
Residual TMP following surgical treatments	5	4	1
TMP after the insertion of a ventilation tube	9	6	3

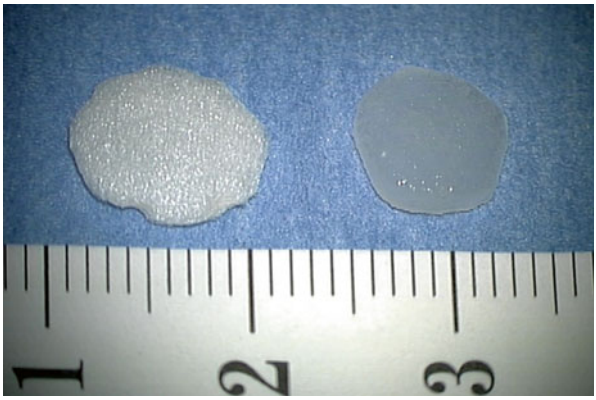

Fig. 4.1 Trimmed gelatin sponge and trimmed gelatin sponge with b-FGF

We limited administration of this treatment to patients with perfect dry TMP and no active inflammation/infection during over 3 years. Temporal bone CTs were analyzed to ensure that all patients had proper aeration without soft tissue density in the mastoid and tympanic cavities prior to treatment.

Patients were classified into three grades based on the size of their TMP: perforations spanning less than 1/3 of the total TM surface area were designated as Grade I (b-FGF, $n=9$; control, $n=2$), from 1/3 to 2/3 as Grade II (b-FGF, $n=25$; control, $n=6$), and over 2/3 as Grade III (b-FGF, $n=19$; control, $n=2$).

Materials used for the TM repair (Fig. 4.1) included gelatin sponge (Spongel: Astellas Pharma Inc. Tokyo, Japan) with basic fibroblast growth factor (b-FGF, Fibrast: Kaken Pharma Co., Ltd Tokyo, Japan) or saline and fibrin glue (Bolheal: KAKETSUKEN, Kumamoto, Japan, Beriplast: CSL Behring K.K., Tokyo, Japan).

4.3 Therapeutic Procedures

The tympanic region was fully anesthetized by applying a cotton-ball soaked in 4 % lidocaine to the perforation in contact with the residual TM for 15 min. Mechanical disruption of the perforation edge was then created under the microscope by a myringotomy knife/micro-pick. A gelatin sponge that was larger than the perforation was immersed in b-FGF (5–30 μg of Trafermin (recombinant human basic FGF) of 100 μg/mL) then inserted into the perforation in contact with the perforation edge of the TM. Fibrin glue was then dripped over the sponge. Figure 4.2 shows these procedures in detail. In cases in which complete closure of the TMP was not achieved, the aforementioned treatment was repeated up to 4 times.

Patients were advised not to blow their nose and/or sniff strongly for a while and were ordered to revisit the hospital 3 weeks after treatment at which point any crust over the TM was removed.

The effectiveness of this treatment for TM repair was evaluated using certain indicators including closure rates, hearing level, sequelae, improvement of tinnitus,

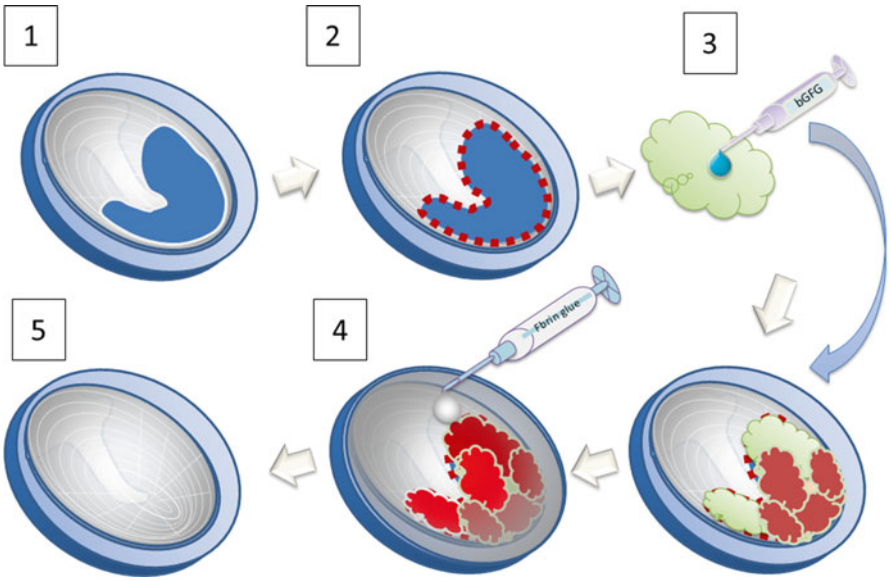

Fig. 4.2 Schematic diagram showing the method and procedures used in this treatment. *1* TM perforation. *2* Following local anesthesia with 4 % lidocaine, a mechanical disruption of the TM perforation edge is created under the microscope. *3* A gelatin sponge immersed in b-FGF is placed over the perforation in contact with the residual TM. *4* Fibrin glue is dripped over the sponge. *5* Three weeks after the treatment, residual crust is removed. In cases of incomplete closure of the TM perforation, the treatment is performed repeatedly

and aural fullness 3 weeks after treatment. The final evaluation was performed 3 months after finishing the treatment. Hearing levels were measured prior to and 3 months following the treatment. The success rate was determined based on the rate of complete closure of the TMP within four courses of treatment. In statistical analysis, Mann-Whiteny U test was used.

4.4 Results

4.4.1 Rates of Complete Closure of the TMPs

The overall rates of complete closure of the TMPs were 98.1 % (52/53) in the b-FGF group and 10.0 % (1/10) in the control group (Table 4.2). In the b-FGF group, the number/rate of the cases in which the TMP was able to close completely after one treatment, two treatments, three treatments, or four treatments were 41 (77.4 %), 7 (13.2 %), 3 (5.7 %), and 1 (1.9 %), respectively. There was only one case in which the TMP failed to close completely by the end of the fourth treatment. Figures 4.3 and 4.4 depict typical cases of perfect TM regeneration. In the control group, one

Table 4.2 Results

Grade	Total $n=53$	Grade I $n=9$	Grade II $n=25$	Grade III $n=19$
Closure rates	98.1 % (52/53)	100 % (9/9)	100 % (25/25)	94.7 % (18/19)
Number of treatment times	1–4 (Avg.:1.4)	1–3 (Avg.:1.3)	1–4 (Avg.:1.4)	1–4 (Avg.:1.6)
Average HL Improvement				
NA	21.7 dB	18.3 dB	21.2 dB	25.8 dB
LA	31.7 dB	28.5 dB	31.7 dB	35.0 dB
Adverse events				
TO	$n=8$	$n=1$	$n=3$	$n=4$
RTM	$n=6$	$n=0$	$n=3$	$n=3$

Grade I: PS (perforation size of TM) <1/3, Grade II: 1/3 < PS < 2/3, Grade III: 2/3 < PS NA: Average hearing level of 0.5, 1 and 2 kHz. LA: Average hearing level of 0.125, 0.25 and 0.5 kHz. *TO* temporary effusion. *RTM* slight retraction of tympanic membrane

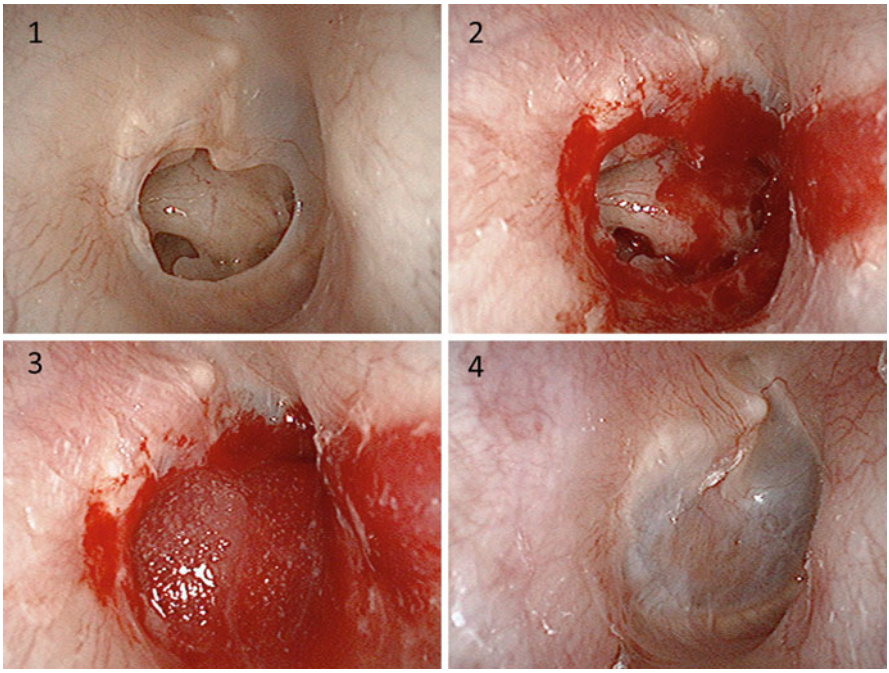

Fig. 4.3 Case 1: A 89-year-old male with chronic otitis media persisting for 50 years. *1* Large, dry, Grade III perforation. *2* Disruption of the perforation edge. *3* Gelatin sponge with b-FGF was placed over the TMP and sealed by fibrin glue. *4* Three months after the treatment the TM was perfectly regenerated. Even in this elderly case, TM can be regenerated by this treatment

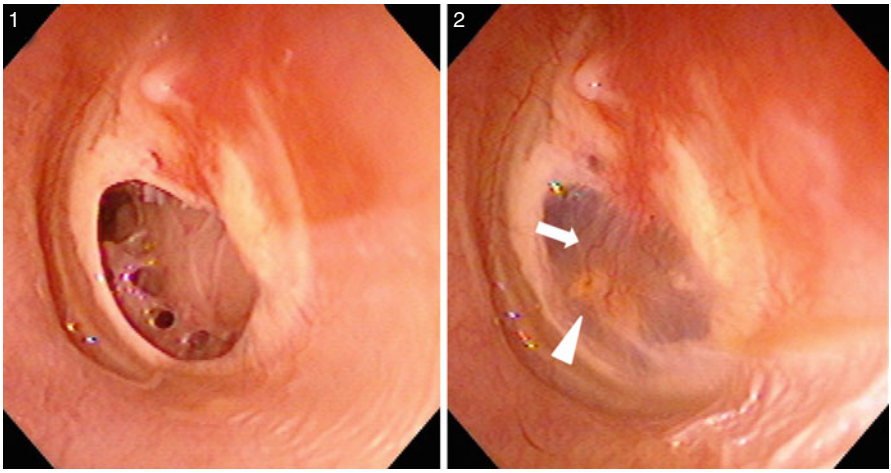

Fig. 4.4 Case 2: A 52-year-old male with otitis media with the TMP. *1* Large Grade II perforation: Almost all remaining TM was accompanied by calcification. *2* Two months after treatment, an almost normal TM with lots of blood capillary (*white arrow*) was regenerated. Remaining gelatin sponge (*white triangle*) was observed in the tympanic side. This will disappear within 3 or 4 months

patient with old traumatic TMP, classified as Grade I, showed complete closure of the TMP after three courses of treatment.

4.4.2 Size of the TMP and Number of Treatments Required

Table 4.2 shows the number of treatments and the presence of any adverse events that occurred during the process of complete TMP repair for each grade of the b-FGF group.

4.4.3 Hearing Improvement

Pure tone audiometry was measured in all patients before and 3 months after treatment. Average hearing levels (average of 0.5, 1, 2 kHz) showed improvement in all patients with successful TM repair in the b-FGF group (Table 4.2). Hearing levels showed remarkable gains in the lower frequency ranges: 0.125, 0.25, and 0.5 kHz (Fig. 4.5).

4.4.4 Tinnitus and Aural Fullness

Prior to treatment, many patients had subjective symptoms. In the b-FGF group, 96.2 % (51/53) of the patients had tinnitus and 86.8 % (46/53) had aural fullness. Of these patients, 98.0 % (50/51) and 95.6 % (44/46) noticed improvements in tinnitus and aural fullness, respectively. In contrast, in the control group, 100 % (10/10) of the patients had tinnitus and 90% (9/10) had aural fullness. Of these, only 10 %

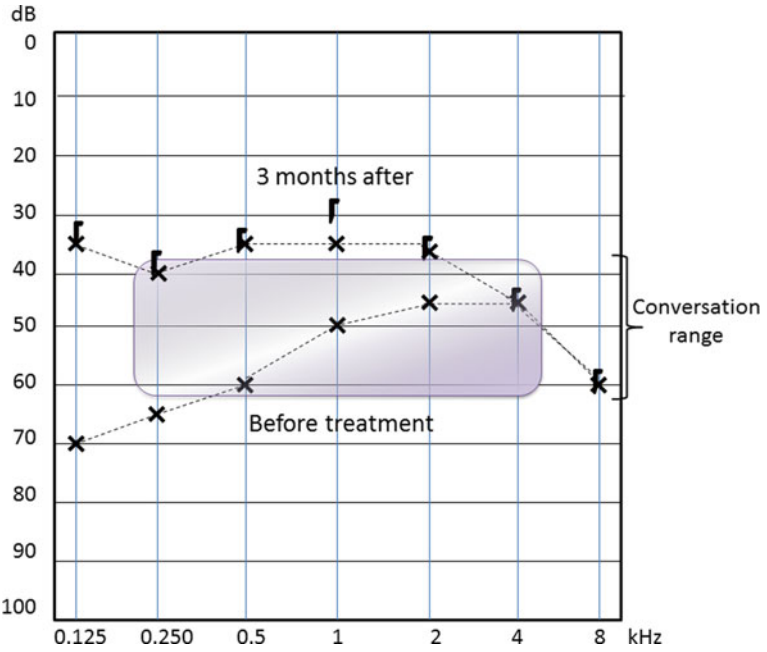

Fig. 4.5 The change in hearing levels in the patient from case 1 (Fig. 4.3), 3 months after treatment with b-FGF. Hearing was measured before and 3 months after treatment. In this case, hearing recovery was mainly observed in the low-frequency ranges

(1/10) and 11.1 % (1/9) of the patients noticed improvements in tinnitus and aural fullness, respectively. Figure 4.6 shows the comparative data.

4.4.5 Time Required to Perform Each Procedure (Excluding Local Anesthesia)

The time needed for each procedure ranged from 7 to 15 min. There were seven cases in which difficulties arose during the treatment. In these seven cases, five cases of Grade II and two cases of Grade III, it was very difficult to directly observe the anterior edge of the perforation because of a protrusion of the anterior wall of the external auditory tube.

4.4.6 Complications

In 8 cases, post-treatment serous otorrhea was observed a few days after treatment. Slight retraction of the TM was observed in 6 cases 3 months after treatment. Two patients with aural fullness had to be treated by puncture of the tympanic membrane. No infection or severe sequela was observed in any patient.

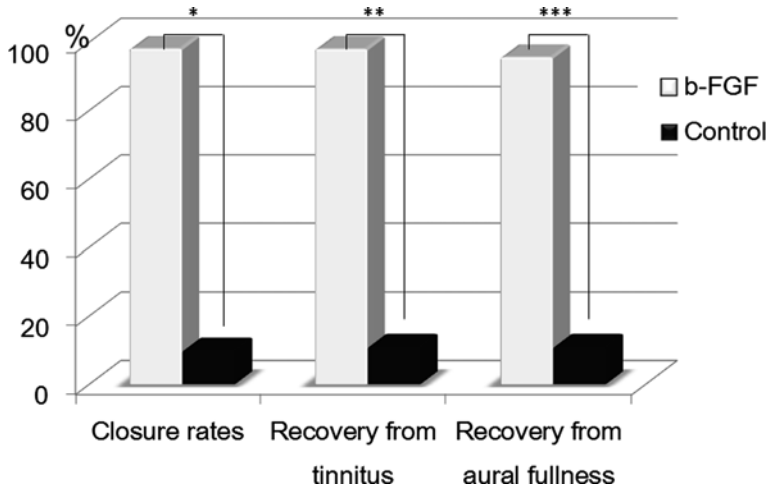

Fig. 4.6 These bar graphs compare the closure rates of TMP, recovery of tinnitus, and aural fullness between the control and b-FGF treatment groups. The b-FGF group showed superior recovery to the control group for all conditions (Mann Whitney's U test: $*p<0.0001$, $**p<0.0001$, $***p<0.0001$)

4.5 Discussion

Tympanic membrane consists of three layers: epithelial layer, intermediate fibrous layer, and mucosal layer. The lateral surface of TM is epithelial layer and is in continuity with the epithelium of external auditory meatus. Mucosal layer is also in continuity with the mucosa of tympanic cavity. Intermediate fibrous layer with capillaries provides TM strength and elasticity. It is impossible to achieve the reconstruction of normal three-layer structures of TM by the conventional surgical treatment such as tympanoplasty/myringoplasty. This is one of the reasons why there is not enough improvement in hearing recovery after these operations. On the contrary, it is possible to achieve the ideal hearing recovery without/with minimum air-bone gap because normal TM with three-layer structures will be able to regenerate by this regenerative treatment.

In patients with TMP, speech articulation and the ability to discriminate sounds can be significantly reduced because of a "cancellation effect" that occurs when sound reaches the oval and round windows simultaneously [2, 3]. Typically, the level of hearing loss is in proportion to the size of the perforation [4]. Large TMPs often cause over 50 dBHL and require a hearing aid to be worn, especially in aged patients. However, the use of a hearing aid can amplify this "cancellation effect" making it quite difficult for these patients to hear. Given these problems, patients would greatly benefit from therapies that would enable the repair of large perforations.

One of the remarkable aspects of the novel regenerative treatment presented here is that patients are able to notice significant improvement in hearing levels immediately following the procedure. The gelatin sponge acts as a pseudo-TM, catching the sound and preventing it from entering the round window, thus eliminating the "cancellation effect".

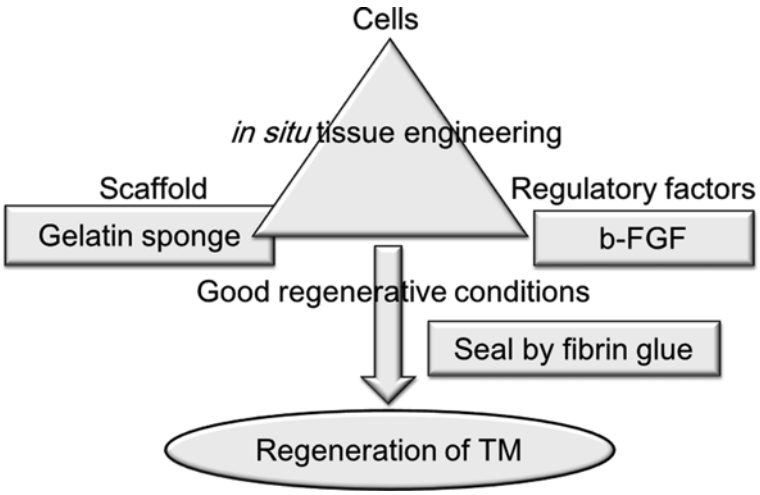

Fig. 4.7 Strategy for regeneration of the TM. In this clinical study, based on the concept of in situ tissue engineering, we introduced just two of the three elements – gelatin sponge for scaffolding and b-FGF as the regulatory factor – as cells were expected to be endogenously supplied from the mechanically disrupted edge of the perforation. To create optimal regenerative conditions, we sealed the gelatin sponge with fibrin glue

In this study, 10 of 12 patients who wore a hearing aid before treatment no longer required its use after regeneration of their TM had been completed. The remaining two patients that continued using a hearing aid were able to hear clearer sound than before treatment.

In addition to these benefits, patients also reported a simultaneous and immediate reduction in tinnitus and aural fullness in almost all cases. These results demonstrate that this treatment has the ability to achieve maximum hearing gains compared to all previously used techniques.

The second remarkable characteristic of this innovative treatment strategy is protection from common infections. Inner ear disorders can often be caused by repeated infections of the middle ear. Malfunction of the inner ear causes not only sensorineural hearing loss but also disequilibrium [5–7]. Moreover, chronic infection of the middle ear may cause degeneration of the chorda tympani nerve resulting in dysgeusia [8, 9].

Surgical treatment is usually required for persistent perforations. As there are numerous disadvantages associated with conventional surgical treatments, much research has been performed to investigate why healing becomes arrested in chronic TMPs and the mechanisms involved in this process [10–12]. Previous studies have demonstrated the ability to repair small chronic TMPs using a collagen sheet [13, 14]; however, for larger perforations spanning greater than 1/3 of the TM, conventional surgical treatment was still required.

According to the principles of in situ tissue engineering, regeneration of tissues/organs requires the introduction of three basic elements – cells, scaffolds, and regulatory factors – under suitable conditions (Fig. 4.7) [15, 16]. In this clinical study, we introduced just two of these elements – scaffolding and a regulatory factor – as

cells were expected to be endogenously supplied from the mechanically disrupted edge of the perforation. That is, mechanical disruption of the perforation edge may stimulate the activity of tissue stem cells of the TM in this region [17–19]. This is another significant advantage of this novel therapy, as cell transplantation would make it difficult to widely and easily apply this therapy as an outpatient treatment.

On the other hand, there is a tendency that complete closure rates of TMP turn down, increasing the number of required treatments. Possible reasons for it are as follows: becoming worse for regenerative conditions and/or exhaustion of tissue stem/progenitor cells by repeating treatments.

We selected this particular combination of a gelatin sponge as a scaffold and b-FGF as a regulatory factor (Figs. 4.7 and 4.1) based on several reports that growth factors and repair materials have been shown to improve TM repair in animal models [13–15, 18, 20–23]. However, the ideal combination of factors and materials for regeneration of the TM has yet to be determined. From the results of our controlled study, a regulatory factor such as b-FGF appears to be a viable option for regeneration of the TM.

There are few growth factors currently available for clinical applications. One of the commercially available growth factors is b-FGF, which is usually used for treatment of skin ulcers or decubitus due to its strong inductive effects on both fibroblasts (from which the name comes) and blood capillaries [21, 22]. This former property makes it particularly suitable for TM regeneration as the par tensa (the largest part of the TM) consists of three layers with the intermediate layer being made of fibrous tissues produced by fibroblasts [20]. Similarly, the capillary-inducing ability of b-FGF may foster improvements in the local regenerative conditions. Consistent with this hypothesis, our study suggests that TM regeneration involves the formation of many new blood capillaries and hypertrophic tissue by 3 weeks posttreatment. Subsequently, the regenerated regions of the TM become thinner and change their shape to more closely resemble normal TM gradually (Figs. 4.3 and 4.4).

Basic-FGF is a polypeptide mitogen that stimulates proliferation of epidermal and connective tissue cells. It is possible that this property of b-FGF may not only stimulate regeneration of the TM but may also stimulate cholesteatoma formation as expression of b-FGF has been reported in cholesteatoma. Friedman et al., however, reported that in an animal model the risk of cholesteatoma formation following administration of b-FGF is minimal when it is applied short term to acute perforations [23]. In our study, no cholesteatoma formation was observed even in the case of chronic TMP. It is possible that b-FGF strongly stimulated regeneration of the intermediate layer comprised of fibrous tissues produced by fibroblasts and thus limited the potential for cholesteatoma formation. Further investigation into this issue will be required to fully evaluate the safety of this treatment.

Given the limitations of two-dimensional sheet-form scaffolds, we selected gelatin sponge as our preferred scaffold material. Gelatin sponge is made of a protein extracted from the collagen found inside the connective tissues of animals and is often used for its hemostatic properties in medical procedures. Gelatin sponge has a high affinity for living tissues and is absorbable in vivo within a few months [24, 25]. Another benefit of gelatin sponge is that it can be cut to fit into any size/shape

Table 4.3 Remarkable advantages of this regenerative therapy

1.	Skin incision and harvesting of autologous tissues are not required.
2.	It can be widely applied to various sizes/shapes of TMPs including total perforations.
3.	It can be performed in 15 min and is a simple/easy outpatient procedure.
4.	Hearing is improved and tinnitus and aural fullness are reduced immediately after the procedure.
5.	Restrictions are not placed on the patient's daily life.
6.	No severe sequelae or other side effects are associated with this procedure.
7.	High success/cure rates and hearing improvements are possible.
8.	It is possible to fully regenerate normal TM morphology.
9.	It is very cost-effective, and minimally invasive.

of TMP and is easy to handle owing to its gel form. Observations of the regenerative processes as it was occurring within the TM revealed that cells will grow into a gelatin sponge and can grow to occupy a large space. Therefore, we designed our treatment such that a portion of the gelatin sponge extends into the tympanic cavity while another portion remains outside of the regenerating TM providing a large surface area for cell growth. Gelatin sponge that remains in the tympanic cavity following TM regeneration is gradually resorbed and disappears within 3 months.

Gelatin sponge works not only as a scaffold for the regeneration of TM but also as a sustained release substrate for b-FGF [26]. These characteristics of gelatin sponge make it particularly advantageous for use in regenerating the TM.

In the final step of our procedure, we seal the gelatin sponge with fibrin glue to insulate it from the outside creating optimal regenerative conditions. Not only does the fibrin glue help to keep the sponge in place, it also prevents cells from drying out and provides a barrier to infection, thus providing ideal culture conditions in vivo.

In this clinical study, we establish a new regenerative therapy for the repair of large and even total TMPs without the need for conventional surgeries. Table 4.3 lists several remarkable advantages of this new therapy. Additionally, this regenerative therapy reduces the psychological, physical, and economical burden on the patient.

The number of the patients who undergo surgical treatment for TMP is estimated to be at least several hundred thousand per year globally. Thus, there exists a huge potential for this new therapy to benefit a large amount of patients worldwide.

4.6 Conclusions

1. We developed an effective new regenerative treatment for perforation of the TM using a combination of b-FGF, gelatin sponge, and fibrin glue without the need for conventional surgical procedures.
2. In this new tissue-engineering therapy, we achieved over a 96 % rate of success.
3. This is an innovative therapy with the added benefits of being an easy, simple, safe, cost-effective, and minimally invasive outpatient procedure.

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Chapter 5
An Approach in Regenerative Medicine for the Treatment of Intractable Otitis Media

A Clinical Study for Regeneration of Mastoid Air Cells Using In Situ Tissue Engineering Techniques

Shin-ichi Kanemaru

Abstract The aim of this clinical study was to develop a new treatment for intractable otitis media such as adhesive otitis media and severe cholesteatoma. Poor development of mastoid cavity and its air cells is often observed in chronic otitis media patients. The functions of mastoid air cells were not known for a long time. Recent studies, however, reported that mastoid air cells have a gas exchange function through blood capillaries in the mucosa that covers the wall of mastoid air cells. This system and the Eustachian tube function to keep the middle ear cavity at an adequate pressure. Therefore, failure of this system is thought to prevent the recovery from chronic otitis media. In this study, we attempted to regenerate pneumatic air cells and their gas exchange function in patients with chronic otitis media.

Eighteen patients (8 males and 10 females) were randomly selected from chronic otitis media patients with cholesteatoma ($n=10$), cholesteatoma with adhesive otitis media ($n=5$), and severe chronic otitis media ($n=3$). Patients ranged in age from 5 to 85 years. One patient with severe chronic otitis media underwent a one-stage surgery. Other patients underwent both stages of a two-stage surgery. These patients were divided into two groups. Group I ($n=5$) underwent regenerative operation and conventional mastoidectomy. Group II ($n=13$) underwent regenerative operation and mastoidectomy with mastoid cortex plasty. The follow-up period ranged from 9 to 12 months after the second stage of tympanoplasty.

Honeycomb-like structures of hydroxyapatite (HA) were made and used as a framework for artificial pneumatic bone. This artificial material with a high macropore ratio of 90 % was mainly composed of calcium phosphate. Its surface was coated with collagen from porcine skin to promote mucosal cell attachment. At the

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first stage of tympanoplasty, collagen-coated HA was implanted into the newly opened mastoid cavity and was affixed with fibrin glue in both groups. After the first surgery, recovery of mastoid aeration and regeneration of pneumatic air cells of the newly opened mastoid cavity were assessed with high-resolution CT scan images.

At the second-stage surgery, the cortical bone of the mastoid was observed to be completely regenerated in all cases in group II. At the second-stage surgery, regeneration of pneumatic air cells in the mastoid cavity was observed in 3 of 17 cases, but in the remaining 14 cases, soft tissues and/or effusion was observed in the mastoid cavity. After removing the obstacles for communication between the mastoid and tympanic cavities, aeration recovered in 14 of 18 cases (77.8 %) and honeycomb-like structures were regenerated in 10 of 18 cases (55.6 %) by the final assessment. In all cases in group I, connective tissues grew into the micropores of the implanted artificial pneumatic bones, and no aeration was observed in the mastoid cavity at the second-stage surgery.

This study demonstrated that the mastoid air cells could be regenerated with implanted artificial pneumatic air cells in the newly opened mastoid cavity in intractable chronic otitis media patients. The mastoid cortex plasty was effective in regeneration of the mastoid air cells because it prevented connective tissue invasion into the mastoid cavity.

This tissue engineering method may be a possible treatment for intractable otitis media because it enables the recovery of gas exchange function of the mastoid air cells.

Keywords Tissue engineering • Regeneration of mastoid air cells • Gas exchange • Hydroxyapatite • Artificial pneumatic air cells

5.1 Introduction

It is needless to say that tympanoplasty is an effective surgical treatment for chronic otitis media. This procedure gives satisfactory short-term results after surgery for many cases of adhesive otitis media and severe cholesteatoma. In long-term observations, there might not be a direct recurrence but gradual changes occur in the newly formed tympanic membrane, tympanic cavity, and mastoid cavity. The morphology of the structure begins to differ considerably from a morphology that can maintain a satisfactory conductive function [1, 2]. The reason is that the structure of the middle ear newly formed by surgery differs greatly from the physiological structure of a normal middle ear. Presently, there is no effective treatment for such intractable otitis media. Thus, current treatment is tympanoplasty to eliminate the lesion.

It is important to have proper pressure control in the middle ear cavity for the maintenance of satisfactory conductive function. In recent years, the Eustachian tube and mastoid air cells have been reported to play a major role in the pressure control in the middle ear cavity [2–11]. That is, gas exchange is performed by the enlarged mucosal capillaries on the surface of the mastoid air cells, and physiological middle-ear pressure is maintained [2, 5–7] (Fig. 5.1).

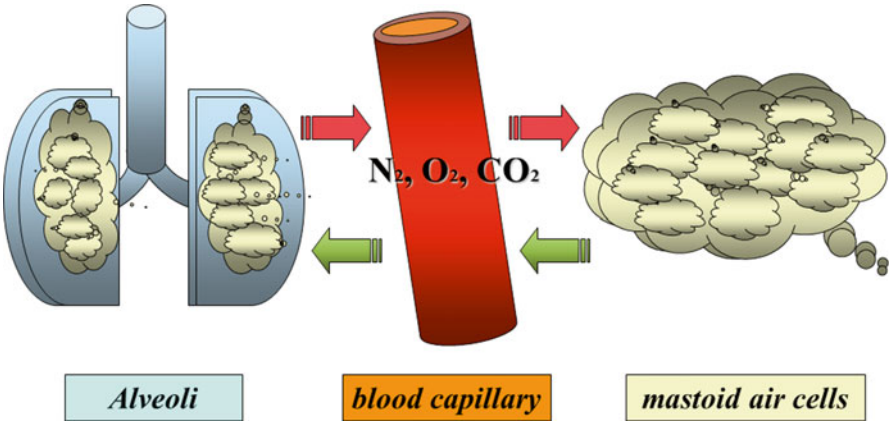

Fig. 5.1 Schematic diagram of gas exchange through capillaries in the mucosa lining mastoid air cells

The main cause of adhesive otitis media and cholesteatoma is considered to be the disruption of pressure control in the middle ear cavity. When a new mastoid cavity is formed by tympanoplasty for intractable otitis media, there is no proper gas exchange function that conventional mastoid air cells have. Thus, the results are poor in many cases.

In this study, we created artificial pneumatic air cells and regenerated mastoid air cells using in situ tissue engineering techniques to develop a treatment for intractable otitis media. In situ tissue engineering is a concept based on regenerative medicine. We report here the results.

5.2 Materials and Methods

5.2.1 Materials

Hydroxyapatite was used as a framework for artificial pneumatic bone. This material had a honeycomb-like structure and a high macropore ratio of 90 %, and was composed mainly of calcium phosphate. Its surface was coated with 1 % collagen derived from porcine skin to produce a basic framework for mastoid air cell regeneration. Collagen (Nippon Meat Packers, Inc. Tsukuba, Japan) was extracted using pepsin treatment from porcine skin, and antigens were removed. Its composition was 70–80 % type I collagen and 20–30 % type III collagen (Fig. 5.2a, b). Collagen used in this study had been subjected to viral inactivation and safety tests and approved in March 2002 by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine. Hydroxyapatite (Pentax, Asahi Optical Co., Ltd. Tokyo, Japan) was the same material used in conventional otologic surgery.

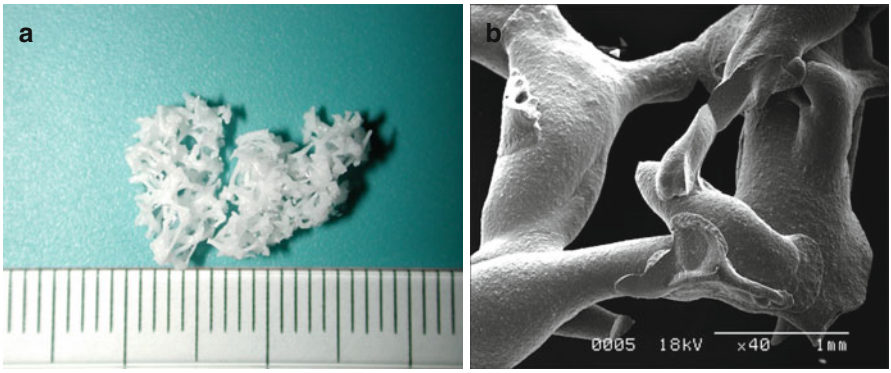

Fig. 5.2 (**a**) Hydroxyapatite coated with collagen. (**b**) Scanning electron micrograph of hydroxyapatite coated with collagen. This hydroxyapatite has a high pore ratio. However, each column of hydroxyapatite has no pore and the structure is very dense

5.2.2 Patients

The subjects of this study were 18 patients with severe chronic otitis media (eight men and ten women; mean age: 54.3 years). There were ten patients with cholesteatoma, five patients with cholesteatoma and adhesive otitis media, and three patients with chronic suppurative otitis media. One-stage surgery was performed in one patient (a 5-year-old girl with chronic suppurative otitis media). All other patients received a two-stage surgery. Only patients who received thorough explanation of the content and goals of this study and who provided consent (or whose family provided consent) were selected for the study.

5.2.3 Surgery

5.2.3.1 First Stage of the Surgery

The treatment in the tympanic cavity is the same as in a conventional surgery.

Opening of Mastoid Cavity and Treatment of Cortical Bone

Conventional mastoidectomy was performed in five patients (group I). In 13 other patients (group II), the cortical bone was removed when the mastoid cavity was opened and the cortical bone was harvested as a bony wall. This bony wall was placed back into its original location after treatment in the mastoid cavity was complete, and reconstruction of the cortical bone wall was performed (Fig. 5.3a, b).

The posterior wall of the external auditory meatus was preserved as is in 4 patients with cholesteatoma and three patients with chronic suppurative otitis media (i.e., closed method). In the remaining patients, the posterior wall was temporarily opened and subsequently reconstructed (canal wall down and canal wall reconstruction).

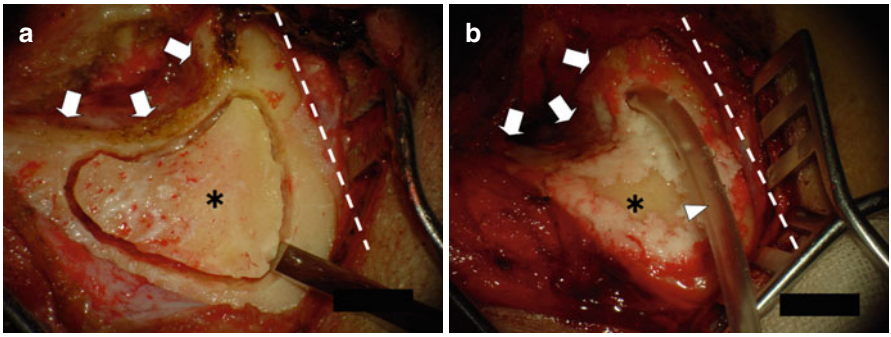

Fig. 5.3 (**a**) Harvesting of cortical bone during antrotomy (first stage of tympanoplasty). For opening the mastoid cavity, a bur was used to make a groove and a flat chisel was used to harvest the cortical bone wall. *White arrow*: posterior wall of the external auditory meatus, *white dotted line*: temporal line, *asterisk*: cortical bone wall. (**b**) Reconstruction of cortical bone wall during antrotomy (first stage of tympanoplasty). *White arrow*: posterior wall of the external auditory meatus, *white dotted line*: temporal line, *asterisk*: cortical bone wall, *white arrow head*: drainage tube

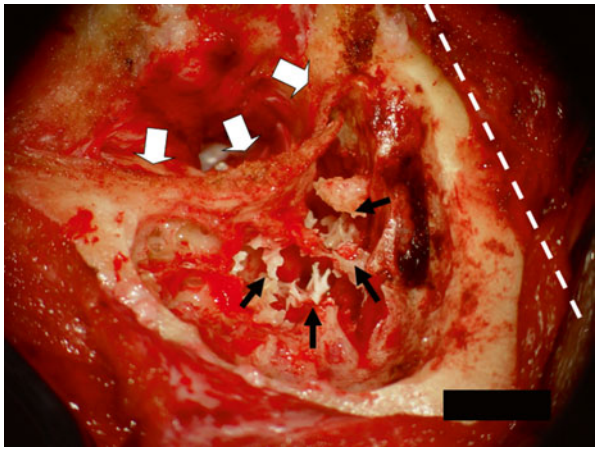

Fig. 5.4 (**a**) Implanted artificial pneumatic bone (first stage of tympanoplasty). At the first stage of tympanoplasty, finely crushed hydroxyapatite was implanted in the opened mastoid cavity. This hydroxyapatite surface was coated with collagen as in Fig. 5.2. *White arrow*: posterior wall of the external auditory meatus, *white dotted line*: temporal line, *black arrow*: implanted artificial pneumatic bone

The reconstructive material for the posterior wall was a bone pate – a mixture of fragments of autologous cortical bone, bone dust collected when the mastoid cavity was opened, and fibrin glue (Bolheal, Fujisawa Pharmaceutical Co., Ltd. Osaka, Japan).

Implantation of Artificial Pneumatic Bone

In the first stage of tympanoplasty, the artificial pneumatic bone was implanted into the opened mastoid cavity (Fig. 5.4). The artificial pneumatic bone was affixed with fibrin glue. A large silicon sheet recommended by Takahashi et al. was placed, extending from the lumen of the tympanic cavity to the mastoid antrum. [10]. Adhesion of the reconstructed tympanic membrane and the promontory was prevented, and a ventilation pathway was established between the Eustachian tube and the mastoid cavity.

In addition, a drain tube of 2–3 mm in diameter was placed to control the pressure in the mastoid cavity and was removed 2–3 weeks after the surgery.

5.2.3.2 Second Stage of the Surgery

The second-stage surgery was performed approximately 9 months (mean) after the completion of the first-stage surgery. In the second stage, the mastoid cavity was reopened. Observations were made on the presence or absence of fluid accumulation, invasion of granulation tissue and connective tissue into the cavity, formation of mucosa in the surroundings of the artificial pneumatic bone, and capillary angiogenesis. Subsequently, removal of granulation tissue, connective tissue, and fluid was performed, if present. If the honeycomb-like structure was formed (even partially), then granulation tissue, connective tissue, and fluid were removed while preserving the formed structure. Opening of the mastoid cavity and reconstruction of the cortical bone were performed using the same method as in the first-stage surgery.

5.2.4 Assessment

Postoperative high-resolution CT scans were used to assess the formation of pneumatic space in the mastoid cavity and the regeneration of the honeycomb-like structure. CT scans were taken three times: before the first surgery and before and after the second-stage surgery. In each patient, the final assessment was made using the last CT images taken. The follow-up period was 9–24 months after the second-stage surgery. (The follow-up period was 12 months after surgery for one patient who did not undergo the second-stage surgery.)

5.3 Results

5.3.1 Regeneration of Cortical Bone

In the second-stage surgery, the posterior meatal wall and cortical bone reconstructed in the first-stage surgery were observed to be almost completely regenerated in all patients (Fig. 5.5).

5.3.2 Regeneration of Mastoid Air Cells

In 3 of 17 patients at the second-stage surgery, regeneration of mastoid air cell structure and regeneration of mucosa, including capillaries, were observed such as shown in Figs. 5.6a, b. However, 14 patients had soft tissue or accumulation of effusion in

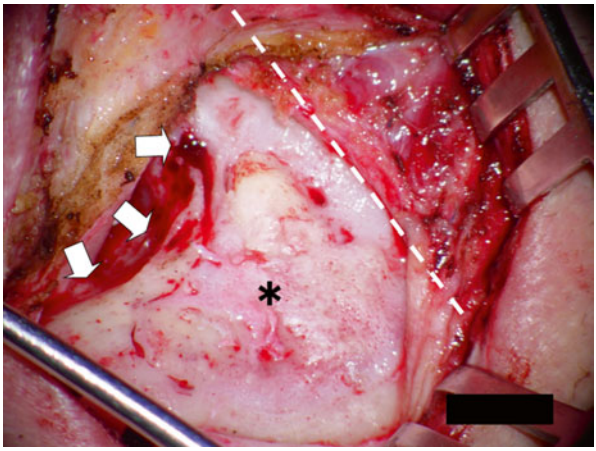

Fig. 5.5 Regenerated cortical bone wall (second stage of tympanoplasty). The findings at the second-stage surgery showed that the cortical bone wall was completely regenerated in the opened mastoid antrum. *White arrow*: posterior wall of the external auditory meatus, *white dotted line*: temporal line, *asterisk*: cortical bone wall

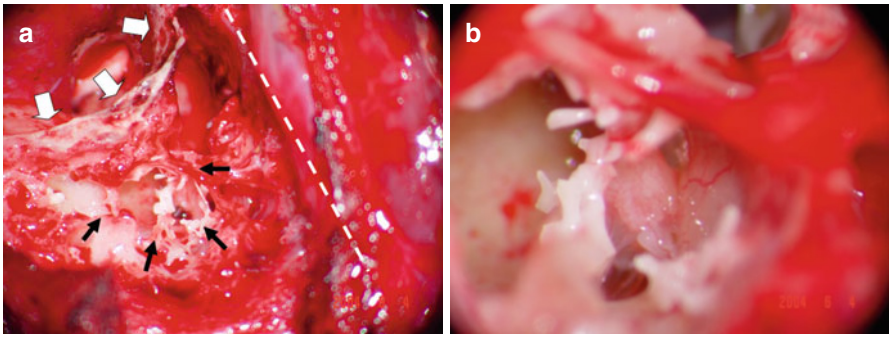

Fig. 5.6 (**a**) Regenerated mastoid air cells (second stage of tympanoplasty). Mastoid air cell structures were formed in the reopened mastoid cavity. (**b**) Regenerated mastoid air cells (second stage of tympanoplasty). This photo is a high magnification image of (**a**), showing formation of mucosa with capillaries in the surrounding of the artificial pneumatic bone. *White arrows*: posterior wall of the external auditory meatus, *white dotted line*: temporal line, *black arrows*: implanted artificial pneumatic bone

the opened mastoid cavity. In most patients, there was a membrane-like matter that blocked the communication between the mastoid antrum and the tympanic cavity in the surrounding of the attic ad antrum, and accumulation of effusion was observed in the mastoid antrum. There was recurrence of cholesteatoma in the attic in one patient. Fourteen of the eighteen patients (77.8 %) showed at least partial aeration from the attic to the mastoid cavity at the final assessment. Ten of the eighteen patients (55.6 %) showed regeneration of the honeycomb-like structure on CT imaging. The remaining four patients showed aeration mainly in the attic. However, there was opacity of soft tissue (and/or fluid) in the surroundings of the artificial pneumatic bone, and a honeycomb-like structure was not formed. In addition, four patients without any aeration showed opacity of soft tissue (and/or fluid) that filled the entire mastoid cavity. Figure 5.7a, b shows images before and after surgery in a patient with regeneration of mastoid air cells.

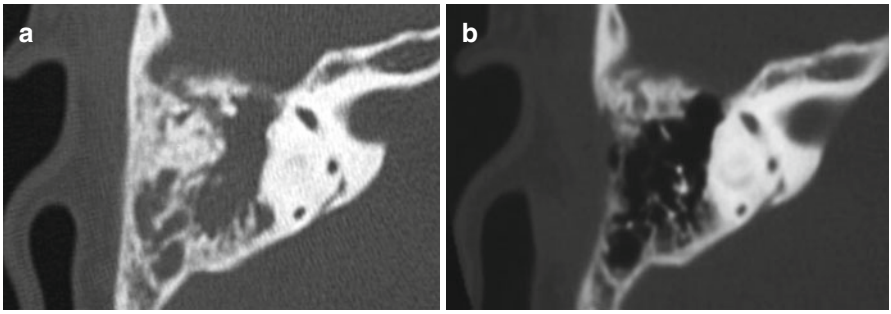

Fig. 5.7 (**a**) Preoperative CT. A 9-year-old girl with adhesive otitis media and cholesteatoma. The image shows opacity of soft tissue that filled the mastoid cavity and no pneumatic space. (**b**) CT taken 6 months after the second-stage surgery. Regeneration of a honeycomb-like structure occurred due to the implanted artificial pneumatic bone, and a sufficient pneumatic space was observed

5.3.3 Relationship Between Regeneration of Cortical Bone and Regeneration of Mastoid Air Cells

At the first-stage surgery after a conventional antrotomy, some patients underwent reconstruction of the cortical bone matching the opening and other patients did not. There were major differences between these patients in the presence of granulation tissue in the opened mastoid cavity, aeration, and regeneration of the honeycomb-like structure. In patients without regeneration of the cortical bone, there was some difference at the second-stage surgery. However, all five patients had invasion of granulation tissue (connective tissue) in the opened mastoid cavity, and this tissue had engulfed the artificial pneumatic bone and occupied the space (Fig. 5.8).

5.4 Discussion

Mastoid air cells have a structure in which there is aggregation of small cavities. These small cavities are made of multiple bony walls and expand in the temporal bone. The surfaces of the bony walls are covered with mucosal epithelium throughout which multiple capillaries are distributed. The physiological functions were not known for a long time for the mastoid antrum (a part of the middle ear much like the tympanic cavity) and mastoid air cells, nearby structures with special morphology. Although the function of mastoid air cells is still not completely known, many studies have shown that they play a role in gas exchange [2–11]. That is, mucosal capillaries on the mastoid air cell surface perform gas exchange depending on gas partial pressure (concentration gradient) in the middle ear cavity. This mechanism is similar to that of the alveolus but not as active as in the alveolus. The gas exchange occurs slowly, in contrast to the very fast opening and closing of the Eustachian tube to control the middle ear pressure [10]. However, the two mechanisms for

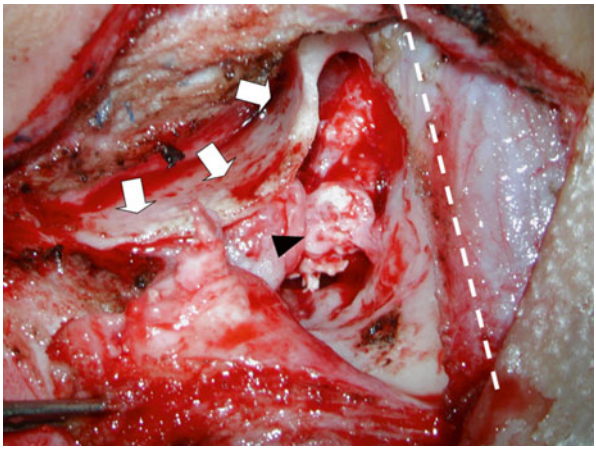

Fig. 5.8 Patient without cortical bone reconstruction (second stage of tympanoplasty). Connective tissue invaded the mastoid cavity and had engulfed the implanted artificial pneumatic bone. There was no regeneration of a honeycomb-like structure. *White arrow*: posterior wall of the external auditory meatus, *white dotted line*: temporal line, *black arrow head*: connective tissue engulfing the artificial pneumatic bone

controlling the middle ear pressure are complementary. Efficient conduction is maintained only when both systems function normally.

Mastoid air cells are not well developed in newborns. As children grow, mastoid air cells enlarge in the temporal bone and continue to develop until the children are approximately 10 years old [12]. If children have repeated bouts of otitis media before 10 years of age (particularly around 4 or 5 years), pathological changes occur in the mucosa in the middle ear cavity and the capacity for gas exchange markedly decreases. The function of the Eustachian tube is not fully developed in childhood. In addition, the accumulated fluid in the middle ear cavity is difficult to be discharged, and loss of pneumatic space hinders gas exchange, resulting in inhibited development of mastoid air cells. Many patients with chronic otitis media have extremely inhibited development of mastoid air cells. This tendency is marked particularly in patients with cholesteatoma or adhesive otitis media. If the development of mastoid air cells is insufficient, the already small pneumatic space is lost due to fluid (e.g., from infection), gas exchange ceases, and the fluid in the pneumatic space is not discharged. A portion of the fluid in the pneumatic space remains as an infectious lesion. Other portions organize and mastoid air cells are lost completely. The middle ear cavity will eventually consist of only a small space, including the tympanic cavity. Maintenance of good conductive function will be difficult, and otitis media will be prolonged because there is no immunity against infection.

The goals of tympanoplasty are to remove the lesion and to restore conductive function. However, the goal of lesion removal cannot be achieved unless the aforementioned pathology is cured. Even if the conduction system is temporarily restored, its long-term maintenance is difficult. Mere modification of surgical procedure will not achieve long-term success, and it will be necessary to change the fundamental concept of treatment.

There has been a remarkable advancement in regenerative medicine in recent years, and new concepts and technologies are continuously emerging. Our study is

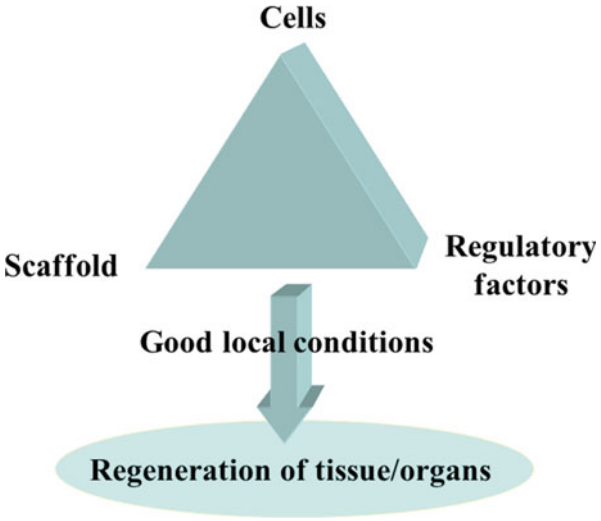

Fig. 5.9 Three elements of tissue engineering

also based on a relatively new concept, in situ tissue engineering. According to a basic concept of regenerative medicine (Fig. 5.9), three elements are necessary in tissue regeneration: cells, scaffold, and regulatory factors. Cells form the basis of regeneration, a scaffold is needed for the growth of cells, and regulatory factors control their growth. Tissue regenerates when these elements are in an appropriate regenerative environment. In the late 1980s, this concept was established and methods were used in which these three elements were placed in an ex vivo environment. The target organ was regenerated to a certain degree and subsequently implanted in a living body. In local tissue regeneration, it was discovered that regeneration can be achieved by placing these three elements directly in a living body from the beginning. Furthermore, it was found that not all three elements have to be supplied from external sources in this method. That is, if an appropriate environment for tissue regeneration is provided, then elements necessary for regeneration are naturally provided by the living body. This concept is that of in situ tissue engineering based on the "field theory" [13–17]. Therefore, an environment favorable to regeneration holds the key to successful tissue regeneration. Based on this concept, we implanted only a scaffold in the opened mastoid cavity in our study.

Our study used hydroxyapatite as a material for artificial pneumatic bone. This hydroxyapatite was composed mainly of calcium phosphate. It is used frequently in otology as an ossicular replacement prosthesis and in orthopedic surgery, dentistry, and oral surgery as a bone substitute, and its safety has been established. The intended use of hydroxyapatite is generally for bone regeneration. Our study did not use hydroxyapatite for bone regeneration but did use it as a scaffold for regeneration of mastoid air cells, that is, regeneration of a functional space. That is, it is a scaffold to expand a place for gas exchange [18, 19]. When the mastoid cavity is opened as a single cavity using a conventional method, the wall surface area is small. Therefore, even if its surface is

covered with mucosa and gas exchange occurs, the capacity for gas exchange is likely insufficient to perform appropriate pressure control in the entire middle ear cavity. For sufficient gas exchange capacity, a mucosal wall surface is necessary, which has the gas exchange capacity of a few dozen to a few hundred times that of a single cavity.

Since hydroxyapatite has an osteoinductive ability, bone is formed if hydroxyapatite is tightly packed into bone defects. In particular, when the pore ratio is high, osteoclasts will more easily penetrate into the pores of hydroxyapatite and the osteogenic ability increases. In contrast, the hydroxyapatite that we designed has a macropore ratio of 90 % based on scanning electron microscopy, but each column has a high density with no pore. This type of hydroxyapatite does not allow osteoclasts to easily penetrate into it and has lower osteoinductive ability than conventional hydroxyapatite. The surface of hydroxyapatite is coated with collagen to promote mucosal cell attachment [19]. The osteogenic ability is lost once the epithelium (including mucosa) covers the hydroxyapatite surface. When hydroxyapatite is implanted, a small amount of it is sparsely placed. Thus, new bone has to fill in the entire area. In the second-stage surgery, we did not find marked new bone formation in the surroundings of hydroxyapatite. As previously mentioned, the mucosa covered the hydroxyapatite surface. In addition, the mucosa was rich in spider-web-like capillaries, extended over a space using hydroxyapatite as a supporting pillar, and expanded the functional surface area for gas exchange. These findings could not be determined on high-resolution CT scans.

In our study, most patients (15/18) had soft tissue or accumulation of effusion in the opened mastoid cavity at the second-stage surgery. However, there was a major difference between patients (10/15) who received cortical bone reconstruction of the opened mastoid cavity and patients (5/15) who did not. In patients with cortical bone reconstruction, accumulation of effusion was observed in the opened mastoid cavity. However, invasion of the soft tissue was rarely seen, and a capillary-rich mucosa covered the hydroxyapatite surface and the walls of the mastoid cavity. The communication with the tympanic cavity was blocked by a membrane-like matter, resulting in the accumulation of effusion. If the communication with the tympanic cavity is reestablished by the removal of this matter, gas exchange begins through the mucosa and a pneumatic space is thought to be formed. The results of our study indicate that more patients have formation of pneumatic space after the second-stage surgery and regeneration of mastoid air cells. However, all five patients without cortical bone reconstruction had invasion of granulation tissue (connective tissue) in the opened mastoid cavity. This tissue had engulfed the artificial pneumatic bone and occupied the space. In other words, invasion of granulation tissue (connective tissue) had taken away a place for mastoid cell regeneration. The above findings indicate that it is important to reconstruct the cortical bone wall of the opened mastoid cavity, because it ensures a space for regeneration to achieve successful mastoid cell regeneration [20].

There are only a small number of cases in which mastoid cell regeneration has been attempted, including cortical bone regeneration. The longest follow-up period has been approximately 2 years since the second-stage surgery. It will be necessary to increase the number of patients and perform long-term follow-up. However, successful mastoid cell regeneration has been achieved with satisfactory pressure

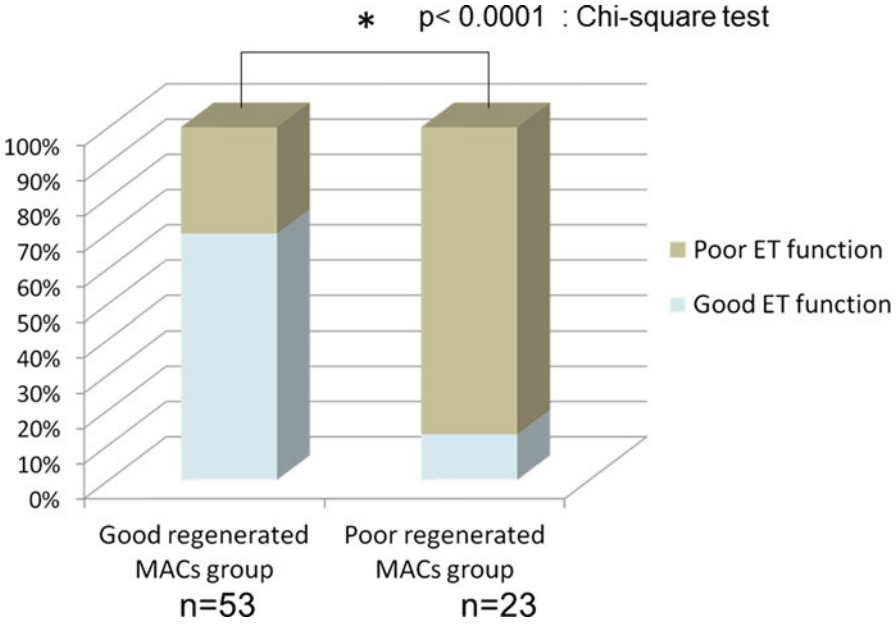

Fig. 5.10 Relationship between MAC regeneration and recovery of ET function. *MAC* mastoid air cell, *ET* eustachian tube. Six months after the second-stage operation, 52.6 % (40/76) of the patients showed improved ET function. Of the 53 cases of good MAC regeneration, 69.8 % (37/53) of the cases showed improved ET function. Of the 23 cases of poor MAC regeneration, 13 % (3/23) of the cases showed improved ET function. The differences between these two groups were statistically significant (*Chi-square test*: $p<0.0001$)

control in the middle ear cavity in patients with adhesive otitis media but without tympanic membrane retraction or adhesion.

Moreover, we performed new clinical study in order to access the ability of regenerated Mastoid air cells to restore normal gas exchange function and contribute to improved ET function [21].

In this study, we found significant improvement in the Eustachian tube function after surgery in many cases of good regeneration of Mastoid air cell; however, improvement was not observed in cases of poor regeneration of Mastoid air cell (Fig. 5.10). This demonstrates that recovery of Mastoid air cell gas exchange function also improves the Eustachian tube function [21]. There are thought to be the mutual-comprehensive relationships between the function of Mastoid air cells and the Eustachian tube.

5.5 Conclusion

This study examined the regeneration of mastoid air cells using in situ tissue engineering techniques. When cortical bone walls were also reconstructed, invasion of granulation tissue and connective tissue was prevented from the external area into the opened mastoid cavity and a space necessary for regeneration was preserved.

The patients with cortical bone reconstruction had better results than patients without reconstruction. Our study showed that regeneration of mastoid air cells has potential as an effective treatment for intractable otitis media.

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Chapter 6
Peripheral Nerve Regeneration by Tissue Engineering for Prevention of Misdirection

Shin-ichi Kanemaru and Tatsuo Nakamura

Abstract We created an artificial nerve conduit called a "polyglycolic acid tube" based on a basic concept of in situ tissue engineering to achieve complete functional regeneration without misdirected reinnervation. We successfully achieved functional regeneration of the recurrent laryngeal nerve in dogs using this artificial nerve conduit. Based on our results, we began its use in human clinical application to regenerate peripheral nerves (such as the recurrent laryngeal nerve, facial nerve, and chorda tympani nerve).

Functional regeneration was observed in the recurrent laryngeal nerve with a shortest defect of 10 mm ($n = 1/6$). Sufficient functional regeneration was observed in cases with a defect of the peripheral branch of the facial nerve: the temporal branch alone or both temporal and zygomatic branches ($n = 2/2$). However, when the defects were in the main trunk of the facial nerve, the recovery was only to a House-Brackmann grade II or III ($n = 4/6$) and misdirected reinnervation could not be prevented. Functional regeneration (sense of taste) was observed in half of the defects of the chorda tympani nerve ($n = 4/8$).

Keywords Tissue engineering • Nerve regeneration • Misdirection • Synkinesis • Artificial nerve conduit

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6.1 Introduction

In the reconstruction of injured nerves, the most effective methods are direct anastomosis and autologous nerve transplantation [1, 2]. Direct anastomosis must be accomplished without excessive tension in the nerve, and its application is limited to cases with a very small gap. In autologous nerve transplantation, the nerves to be reconstructed are often motor nerves. Sensory nerves are often harvested as a graft material, and it is important to select a nerve with the appropriate diameter. When a sensory nerve is resected to be used as a reconstructive material, an inevitable sequela is anesthesia in the areas innervated by that nerve. If multiple nerves need to be reconstructed, a wide extent of nerves must be harvested and there will be an extensive sequela. Furthermore, a nerve mass can develop at the site of graft anastomosis and some cases require another neurectomy due to pain. In addition, the recovery of motor function is not significant for autologous nerve transplantation. In particular, satisfactory results in regeneration cannot be expected in the recurrent laryngeal nerve [3–8] and the facial nerve [9–12] in which misdirected reinnervation is likely. Reconstruction of these nerves is performed not necessarily for functional recovery but for prevention of atrophy of muscles innervated by these nerves.

6.2 Regenerative Process of Peripheral Nerves

When a peripheral nerve is transected, the injury signal is transmitted to proximal cell bodies and regeneration begins. The nodes of Ranvier enlarge in the transected nerve stump within a few hours after transection, and sprouting occurs from the tip of the enlarged area. Naked axons with a growth cone at their leading edge elongate to the periphery. Subsequently, Schwann cells migrate along the regenerated axons and lead the elongation of the nerve fibers while releasing various factors. In the process of nerve regeneration, it is most important for the sprouting growth cone and subsequent elongating axons to reach their peripheral target organ in a short period of time without interference.

Data from a previous animal experiment have shown that a nerve mass forms at the site of reconstruction in a large proportion of animals after autologous nerve transplantation [13] (Fig. 6.1). The reason is that the transplanted nerves underwent Wallerian degeneration, and regeneration started from the proximal nerve stump before a path for regenerated axons was provided. Thus, the transplanted nerve likely obstructed the path for elongation of the regenerated axons. That is, a mass was formed in the surroundings by the regenerated axons with nowhere to go and by subsequent Schwann cells and nerve fibers. In such an environment, there are very few nerve fibers that reach the target organ. Even if the nerve fibers reach the organ, it will take a very long time. Thus, atrophy of the target organ can occur and func-

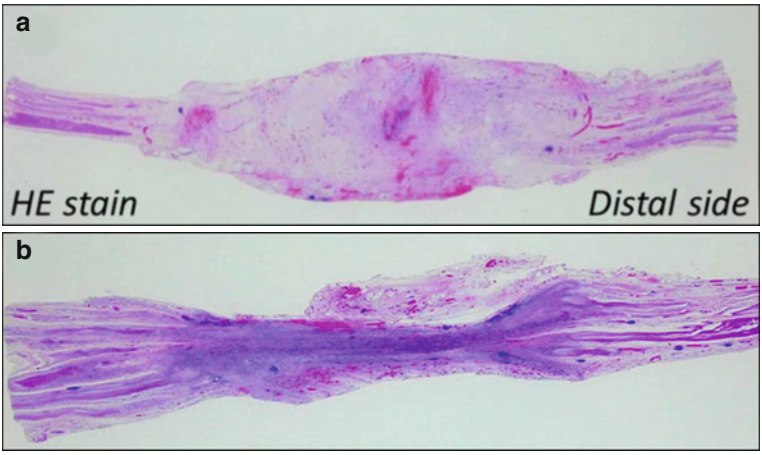

Fig. 6.1 (**a**) Auto-nerve graft. Eight weeks after operation, 60 % cases had developed a neuroma at the autologous graft site [13]. (**b**) PGA tube implantation. Eight weeks after operation, the gap was filled with nerve fibers and no neuromas were observed in all cases [13]

tional recovery might not be possible due to misdirected reinnervation. In particular, the recurrent laryngeal nerve has conditions unfavorable to regeneration: (1) it has a very small diameter, (2) it travels a long distance from the brain to its target organ, (3) its nerve bundle includes nerve fibers that innervate antagonistic movements of muscles controlling the opening and closing the vocal cords, and (4) its target organ (muscles) is very small.

Uno et al. [14] used rats and examined the metabolic activities in proximal cell bodies that received the signal caused by peripheral nerve injury. They focused on the changes in the production of growth factor associated protein-43 (GAP-43), which is thought to play an important role in regeneration. The study showed that GAP-43 mRNA expression increased in the cell bodies of the nucleus ambiguus motor neurons after recurrent laryngeal nerve transection. The expression peaked at 1 week after transection and began to decrease at approximately 2 weeks after transection. This peak period of GAP-43 mRNA expression was different from that in the nuclei of other peripheral nerves after transection. The regenerative ability of the laryngeal nerve was much lower than that of other nerves.

6.3 Artificial Nerve Conduit

In the regenerative process of injured peripheral nerves, an environment favorable to regeneration is important at as early a stage as possible to achieve good results. Thus, we created an artificial nerve conduit, a polyglycolic acid (PGA) tube, based on a basic concept of in situ tissue engineering [15]. The surface of this tube is

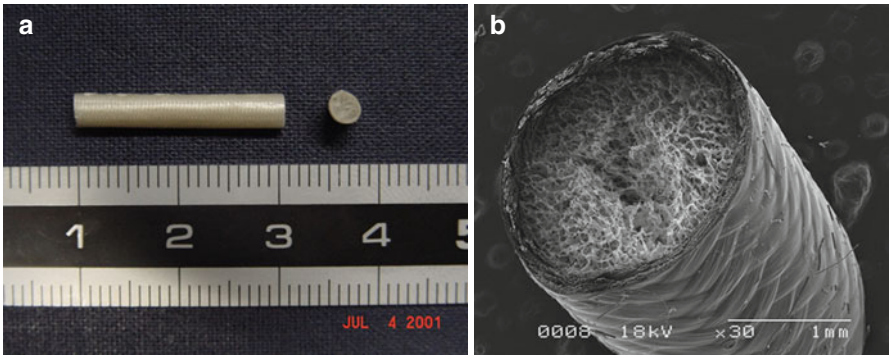

Fig. 6.2 Artificial nerve conduit. (**a**) Polyglycolic acid (PGA) tube in longitudinal and cross-sectional views. (**b**) Image of PGA tube by scanning electrochemical microscope. The outer and inner surfaces are coated with amorphous collagen layers and filling its interior with a collagen sponge

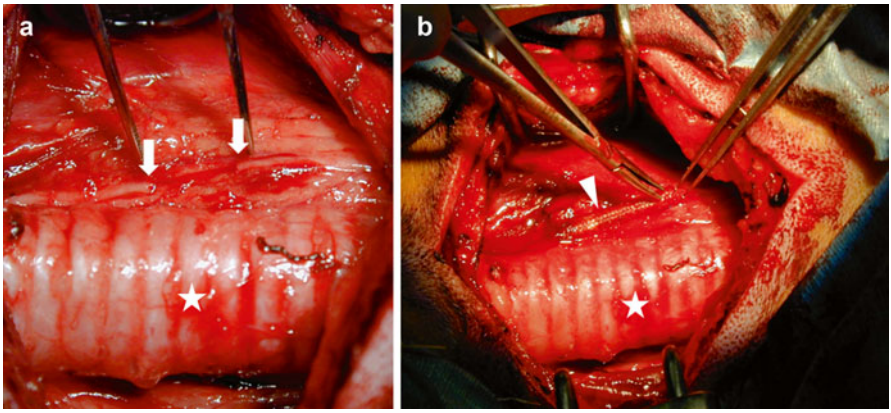

Fig. 6.3 Intraoperative views of canine model. (**a**) After resection of RLN, (**b**) Reconstruction of nerve defect by PGA tube. *White arrow*: Recurrent laryngeal nerve (RLN) ends (10 mm defect); *white arrow head*: Reconstruction of RLN by PGA tube; *white asterisk*: Trachea

covered with a mixture of porcine collagen type I (70 %) and collagen type III (30 %). A collagen had low antigenicity due to removal of telopeptide. The tube was then filled with the same collagen and treated dehydrothermally at 140°C at a pressure of 10 Pa for 24 h in order to cross-link the collagen molecules. PGA is a high-molecular-weight compound with the simplest aliphatic polyester polymer. It is hydrolyzed in a living body and completely degrades in approximately 4 months. On the other hand, it maintains its shape for a certain period of time.

We have prepared several types of PGA tubes to date and conducted experiments on them. With a goal of clinical application, we created the current type that can be mass produced and has superior physical strength (Fig. 6.2).

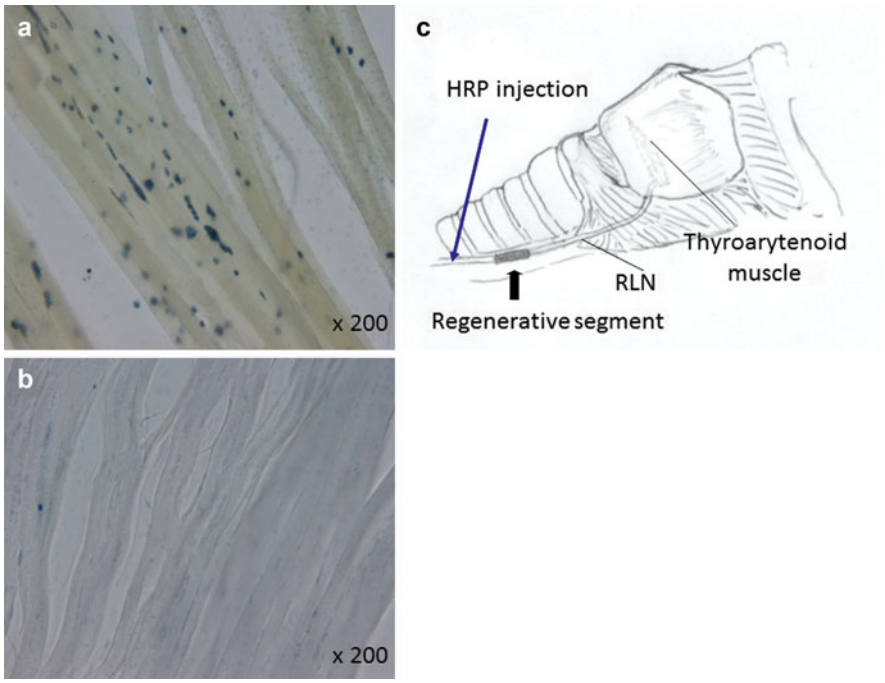

Fig. 6.4 Recovery of the axonal transport of severed recurrent laryngeal nerve (RLN). (**c**) At 6 months after operation, wheat germ agglutinin-horseradish peroxidase (HRP) was injected on the proximal side of the reconstructed RLN. Nerve terminals in cricoarytenoid muscles were stained with HRP on PGA tube side (**a**) but were not stained on auto-nerve implantation side (**b**)

Studies have been conducted on rats, cats, and dogs to regenerate peripheral nerves after experimental resection. PGA tubes filled with collagen were used in these studies. The results in dogs indicated that the recurrent laryngeal nerve can have functional regeneration after resection [9, 11] (Figs. 6.3, 6.4, and 6.5).

6.4 Clinical Application of PGA Tube for Nerve Regeneration

Based on the aforementioned research results, we began to use the PGA tube in clinical application for nerve regeneration in 2002 after the review and approval by the ethics committees of the Department of Otolaryngology, Head and Neck Surgery, at Kyoto University Hospital in Kyoto and the Department of Otolaryngology, Head and Neck Surgery, at Kitano Hospital in Osaka, Japan.

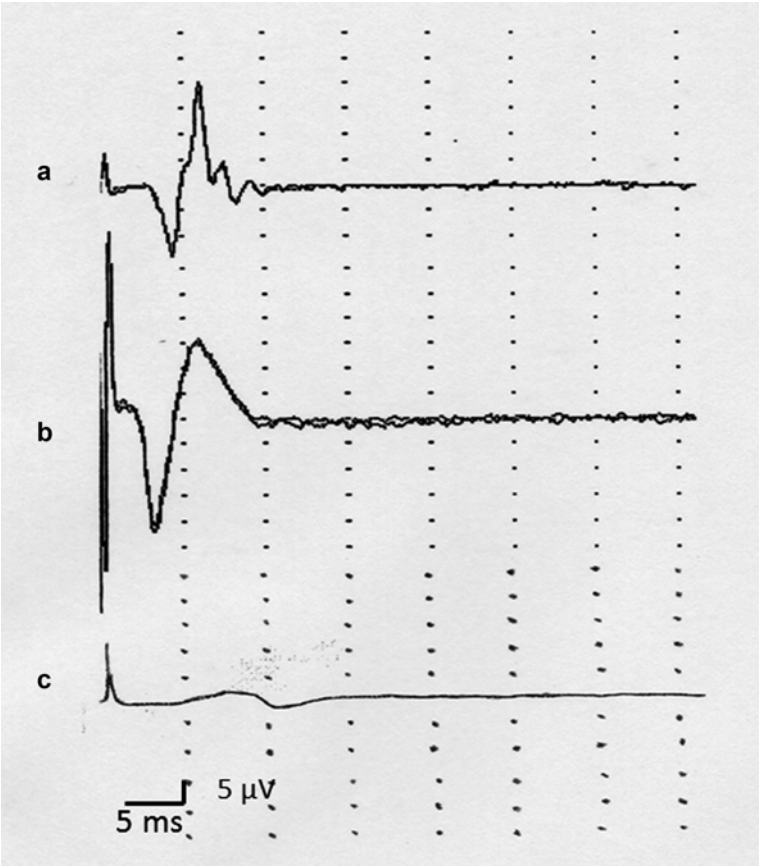

Fig. 6.5 Electrophysiological assessment: Compound muscle action potentials (CMAPs) from cricoarytenoid muscles, 6 months after operation. (**a**) PGA tube reconstructed side, (**b**) nonoperated side (normal control), (**c**) auto-nerve graft side

6.4.1 Recurrent Laryngeal Nerve

As mentioned earlier, the recurrent laryngeal nerve is a nerve whose functional regeneration is most difficult to achieve. We have used PGA tubes in six patients to date, of whom only one showed movement of the vocal cords. This patient was a 54-year-old man who had laryngeal cancer with primary glottic cancer. He had undergone radiation therapy and laser surgery and achieved local control. However, he had cervical metastasis with invasion of the recurrent laryngeal nerve. Therefore, 1 cm of the right recurrent laryngeal nerve was resected and a PGA tube was used for nerve regeneration. Three months after regeneration, the patient had vocal cord movements and functional regeneration of the recurrent laryngeal nerve.

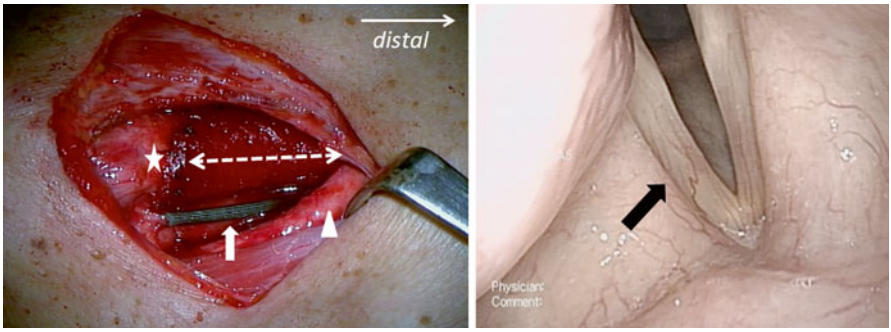

Fig. 6.6 A case of simultaneous regeneration of the trachea and recurrent laryngeal nerve (human: thyroid cancer T4NxMo). *Star*: cranial trachea; *arrowhead*: common carotid artery; *dotted double-headed arrow*: implanted tracheal prosthesis; *single-headed white arrow*: area of the recurrent laryngeal nerve (5 cm) reconstructed using an artificial nerve conduit (PGA tube); *single-headed black arrow*: vocal fold in regenerated side. (**a**) After suture fixation of the tracheal prosthesis and artificial nerve conduit in the area of the tracheal defect and in the area of recurrent laryngeal nerve resection, respectively. (**b**) Two years after the operation, though movement of the vocal fold in regenerated side did not recover, no atrophy of vocal fold in regenerated side was observed

In the other five patients, 2 cm or more of the recurrent laryngeal nerve had been resected and no functional regeneration was observed. However, all five patients had satisfactory vocalization. Although there was no functional regeneration due to misdirected reinnervation, it was speculated that regeneration of the recurrent laryngeal nerve with a PGA tube was useful in preventing the atrophy of the laryngeal muscles innervated by the nerve. Figure 6.6 shows a case of simultaneous regeneration of the trachea and recurrent laryngeal nerve (thyroid cancer T4NxMo). In this case, functional regeneration of the recurrent laryngeal nerve was not observed. The patient, however, keeps good voice because of no atrophy of the vocal fold.

6.4.2 Facial Nerve

As in the recurrent laryngeal nerve, synkinesis due to misdirected reinnervation occurs very frequently for the facial nerve. For example, the following types of sequela are frequently observed. Tearing of the eyes can occur during a meal when there is misdirected reinnervation. In such a case, the nerve fibers destined for the salivary glands are misdirected to the lacrimal gland (a phenomenon called "crocodile tears"). Misdirected reinnervation can also involve the facial nerve temporal/zygomatic branches and the mandibular/buccal branches where the corners of the mouth move with the upward and downward movements of the eyebrows or with the closing of the eyes. Patients with facial nerve disorder are subjects for nerve regeneration if their facial nerves were transected at surgery or by trauma. All of our clinical cases (eight patients) fit this category.

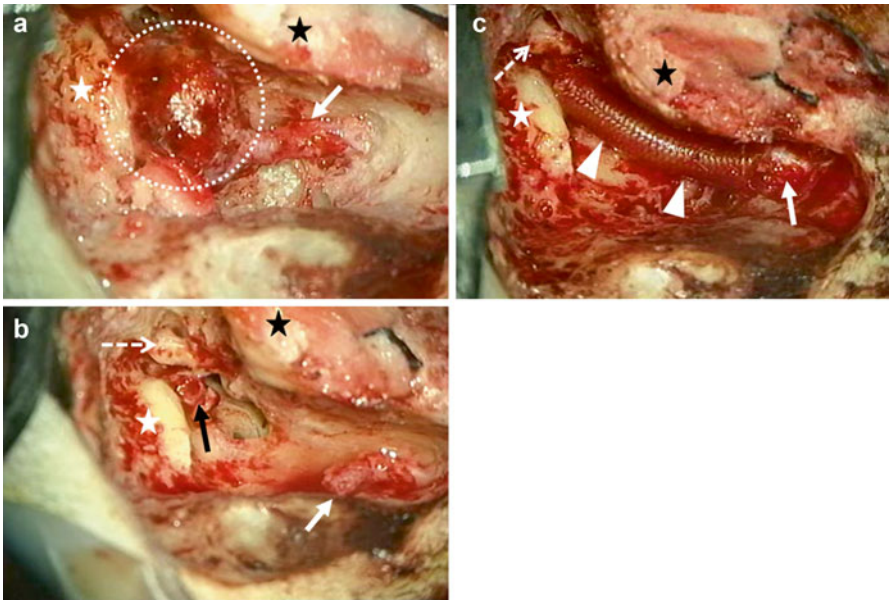

Fig. 6.7 Intraoperative views of human model: facial nerve schwannoma. (**a**) Before resection of tumor, (**b**) After resection tumor, (**c**) Reconstruction of nerve defect by PGA tube. *White arrow*: Mastoid portion of facial nerve; *white dotted arrow*: incus, *white arrow head*: reconstruction of facial nerve by PGA tube; *white asterisk*: lateral semicircular canal; *black asterisk*: posterior wall of external auditory canal; *white dotted circle*: tumor

Two patients injured a particular branch (or particular branches) of the facial nerve due to trauma, and both patients achieved complete recovery after nerve regeneration using a PGA tube. Both patients suffered nerve injury due to traffic accidents, and one patient severed the temporal branch alone and the other patient severed the temporal/zygomatic branches. The treatment was performed 3 months after each accident.

The other six patients had damage to the main trunk of the facial nerve: three patients with schwannoma (Fig. 6.7), one patient with nerve transection due to medical error at another hospital (occurred 6 months prior to presentation at our hospital), and two patients with parotid gland cancer. All six patients eventually recovered to a House-Brackmann grade II or III, synkinesis remained, and complete recovery was not achieved.

6.4.3 Chorda Tympani Nerve

There were a total of eight patients with damage to the chorda tympani nerve: due to invasion by cholesteatoma in six patients and due to nerve transection caused by a medical error during a middle-ear surgery in two patients. The evaluation of

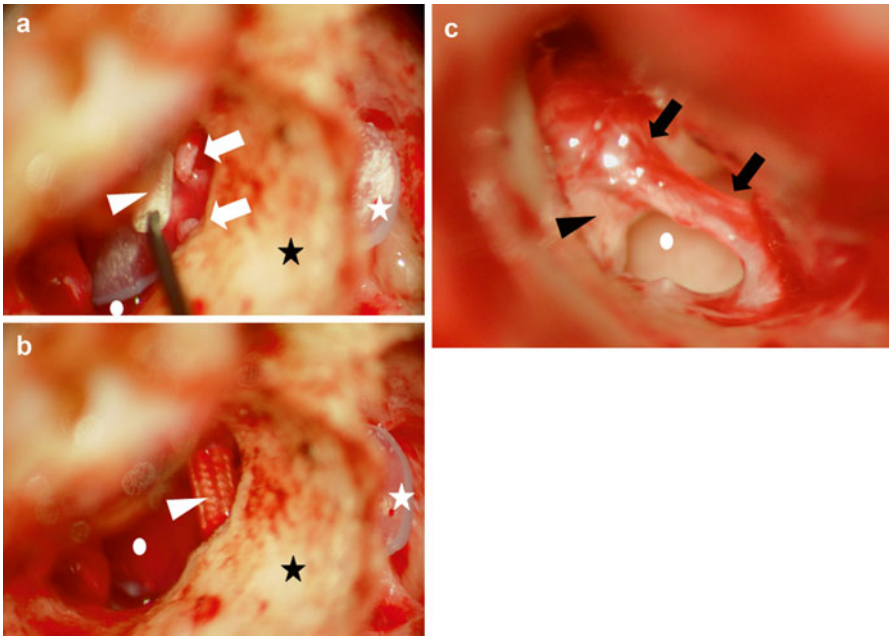

Fig. 6.8 Intraoperative views of human model: reconstruction of chorda tympani nerve (CTN) by PGA tube after resection of CTN (**a**, **b**), (**c**) One year after the first operation, regenerated CTN was observed. *White arrow*: nerve ends; *white arrow head*: PGA tube; *white asterisk*: silicon wafer; *black asterisk*: posterior wall of external auditory canal; *white circle*: tympanic cavity; *black arrow*: regenerated chorda tympani nerve; *black arrow head*: stapes

chorda tympani nerve regeneration was performed preoperatively and 1 year postoperatively using electrogustometry. Since the assessment of sense of taste by electrical stimulation is subjective and can be inconsistent, multiple measurements were made and their mean value was calculated. The results showed that the sense of taste after surgery recovered to the level before the surgery in four patients, the sense of taste recovered slightly in two patients, and the sense of taste did not recover in two patients (Fig. 6.8).

6.5 Conclusion

To date, most of our clinical cases of peripheral nerve regeneration using a PGA tube showed functional recovery of one target organ. In the nerve regenerative process, it seems certain that the PGA tube prevents the invasion of tissue from the outside and plays its most important role as a scaffold for elongation of axon and growth cone and for subsequent migration of Schwann cells. However, function has not been restored if the criterion is accuracy of innervation of multiple muscles (target organs to be reached through axonal regeneration). Although the hurdle is

high for complete recovery, the results have shown success at least to the level of autologous nerve transplantation. To achieve complete recovery without misdirected reinnervation, it is necessary to conduct basic research on retrograde signaling from the target organ in addition to improvement of the tube and administration of cellular and growth factors.

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Part III
Development and Regeneration of the Nose and the Paranasal Sinuses

Chapter 7
Development and Regeneration of the Nose and the Paranasal Sinuses

Atsushi Suehiro and Toshio Ueda

Abstract In the first section of this chapter, nasal embryology is described. An understanding of nasal embryology and anatomy is of considerable importance for regenerative medicine of the nose. Most of the components of the nasal and paranasal sinuses arise from the ectodermal germ layer. During the fifth embryonic week, nasal development starts from a small pit, which subsequently deepens to form a cavity. Formation of the soft and hard palate, and fusion of the nasal septum and the palate are completed in the twelfth embryonic week. Differentiation of the nasal respiratory epithelium is almost finished by the twenty-fourth embryonic week. In the second section of this chapter, progress in tissue engineering techniques for nasal reconstruction is summarized. Tissue engineering of the mucosa, cartilage, bone, and skin is essential for complete nasal reconstruction. The final section describes the use of regenerative medicine in restoring olfactory function. The olfactory system has a high intrinsic regenerative capacity; however, many patients experience olfactory disorders. Recent advances in the field of olfactory regenerative medicine are explained.

Keywords Rhinology • Nasal embryology • Tissue engineering • Regenerative medicine

7.1 Embryology

7.1.1 Introduction

The nasal and paranasal cavities have a complex structure, being surrounded by the skull base, the orbital bones, and the oral cavity. Therefore, a small abnormality easily causes eating or speech disorders. To treat these disorders, an understanding

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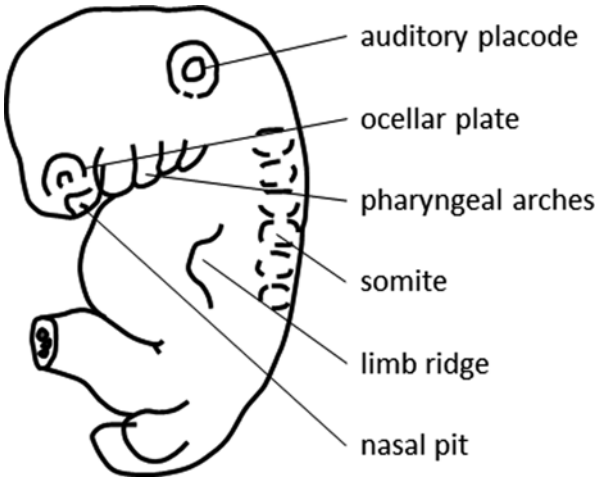

Fig. 7.1 The fourth-week embryo

of the embryology of the nasal and paranasal sinuses is necessary. In the following chapters, nasal embryology is described in chronological order, from the time of fertilization to adulthood [1, 2].

7.1.2 Pre-embryonic Period

Three days after fertilization, the zygote divides into a 16-cell morula. The morula is composed of an inner cell mass and an outer cell mass. As the morula migrates into the uterine cavity, a blastocele forms around the inner cell mass. At this stage, the embryo is called a blastocyte. Implantation occurs at the end of the first week. The inner cell mass differentiates into the epiblast and hypoblast, and these two layers form the bilaminar germ disc. During the third week, epiblast cells in the region of the primitive streak invaginate between the epiblast and hypoblast layers, forming the third layer. This structure, consisting of the endodermal, mesodermal, and ectodermal germ layers, is called the trilaminar germ disc. The nasal and paranasal components primarily develop from the ectodermal germ layer.

7.1.3 Embryonic Period

7.1.3.1 Fourth Week

At the beginning of the fourth embryonic week, four pairs of pharyngeal arches are formed (Figs. 7.1 and 7.2). By the end of the fourth week, facial prominences, which are mainly derived from the first pair of pharyngeal arches, clearly appear. Maxillary prominences are identifiable bilateral to the stomodeum, and mandibular prominences are identifiable caudal to the stomodeum. The frontonasal prominence,

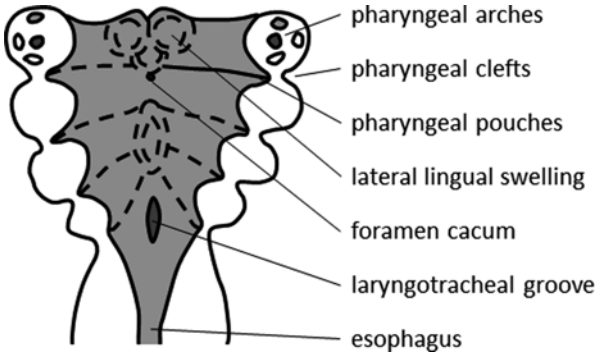

Fig. 7.2 The pharyngeal arches and clefts

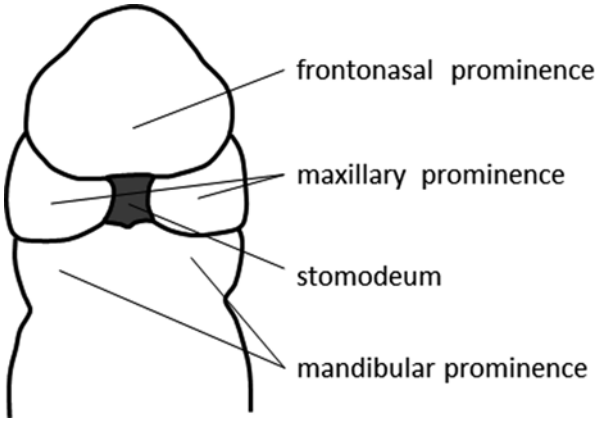

Fig. 7.3 Frontal view of the embryo at the beginning of fourth week

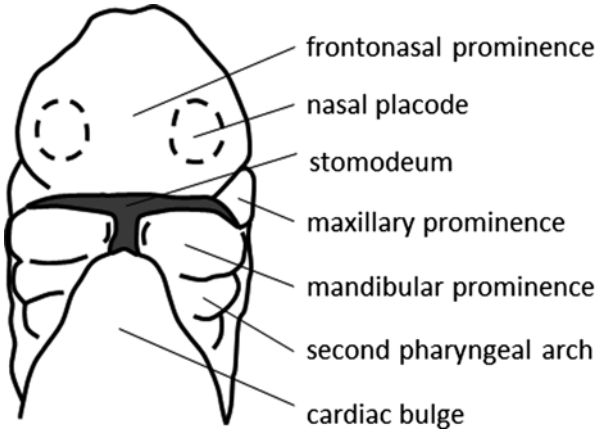

Fig. 7.4 Frontal view of the embryo at the end of fourth week

which is formed by the proliferation of mesenchymal cells, constitutes the upper border of the stomodeum (Fig. 7.3). At the caudal side of the frontonasal prominence, a pair of nasal placodes develops from the thickening surface ectoderm (Fig. 7.4).

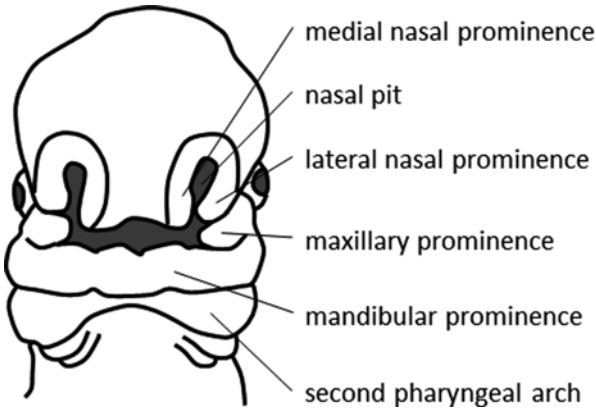

Fig. 7.5 Frontal view of the embryo in the fifth week

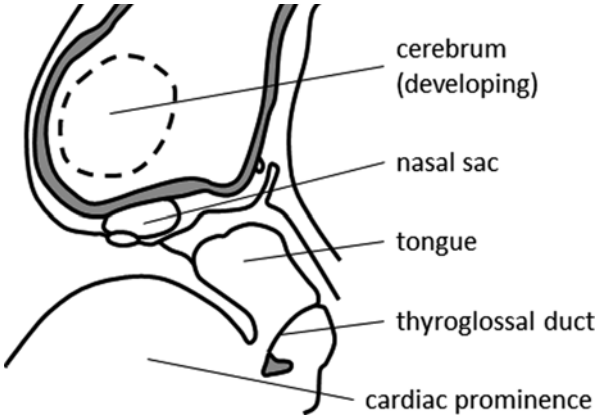

Fig. 7.6 Sagittal section of the embryo in the fifth week

7.1.3.2 Fifth Week

In the fifth embryonic week, the nasal placodes become hollow and form nasal pits. The inner and outer edges of the nasal pit are called the medial and lateral nasal prominences, respectively. As these two prominences grow, the nasal pits become deeper (Fig. 7.5) and develop into the nasal sac, which ultimately becomes the nasal cavity (Fig. 7.6). Subsequently, the maxillary prominences grow medially and compress the medial nasal prominences. The cleft between the medial nasal prominence and the maxillary prominence disappears, and the medial nasal prominences fuse at the midline to form the intermaxillary segment.

7.1.3.3 Sixth Week

At the beginning of the sixth week, the maxillary prominences and the lateral nasal prominences are separated by the nasolacrimal groove. The surface ectodermal layers of these two prominences fuse, forming a groove, and subsequently, a canal

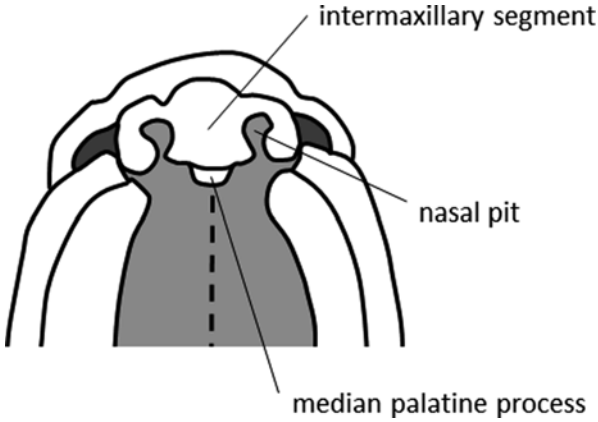

Fig. 7.7 Upward view of the palate at the beginning of sixth week

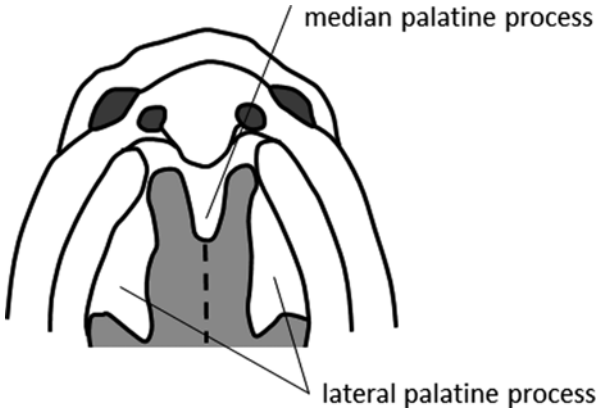

Fig. 7.8 Upward view of the palate at the end of sixth week

known as the nasolacrimal duct. The upper end of the nasolacrimal duct widens to form the lacrimal sac. At the deeper layer, the primary palate is derived from the intermaxillary segment (Fig. 7.7). The dorsal region of the frontonasal prominence grows longitudinally, later becoming the nasal septum. Also, the lateral palatine process starts to expand (Fig. 7.8).

7.1.3.4 Seventh Week

During the fifth and sixth embryonic weeks, the nasal pits invaginate deeper into the underlying mesenchyme. The oronasal membrane initially separates the pits from the oral cavity (Fig. 7.9). Subsequently, in the seventh week, the membrane is penetrated and the primitive choanae are formed. At the end of seventh week, the primitive choanae divide the nasal cavity and the pharynx, and the nasal meatus plug closes the external nares. Distinct areas of the face develop from each of the facial prominences: the forehead and dorsum, apex, and nose root from the frontonasal prominence, the nasal wing from the lateral nasal prominence, the nasal septum and

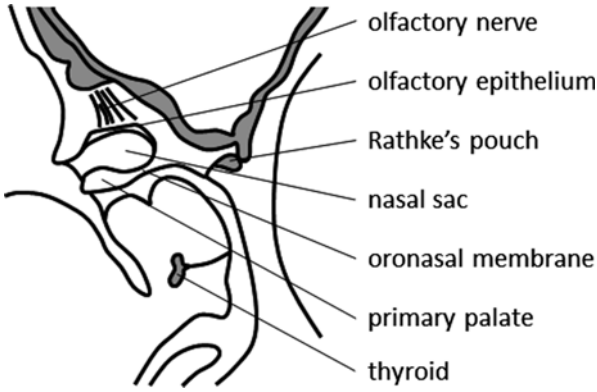

Fig. 7.9 Sagittal section of the embryo in the seventh week

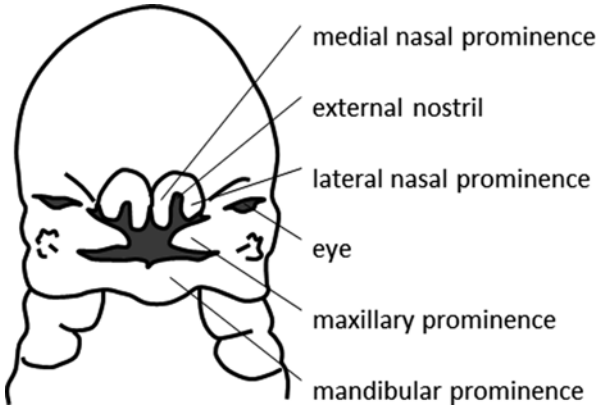

Fig. 7.10 Frontal view of the embryo in the eighth week

philtrum from the medial nasal prominence, the top half of the cheek and upper lip from the maxillary prominence, and the bottom half of the cheek, lower lip, and chin from the mandibular prominence. At this time, the nasal cavity is covered by undifferentiated stratified cuboidal epithelium (Fig. 7.10).

7.1.4 Fetal Period

7.1.4.1 Eighth Week

The nasal sac, a cartilaginous tissue that spans from the nasal septal area and the ethmoid bone to the inferior nasal conchae, is the origin of the supportive tissues of the nasal and paranasal sinuses. The nasal sac extends to the sphenoid bone at posterior part of the nasal cavity (Fig. 7.11). From the lateral walls, three pairs of nasal conchae form the preturbinates. During this time period, the majority of the nasal cavity epithelium consists of undifferentiated or nonciliated cells. However,

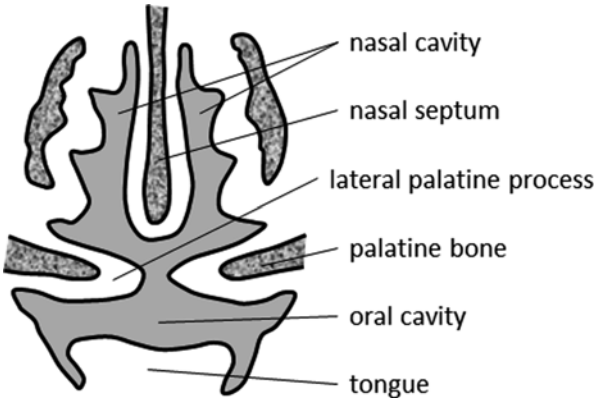

Fig. 7.11 Coronal section of the embryo in the eighth week

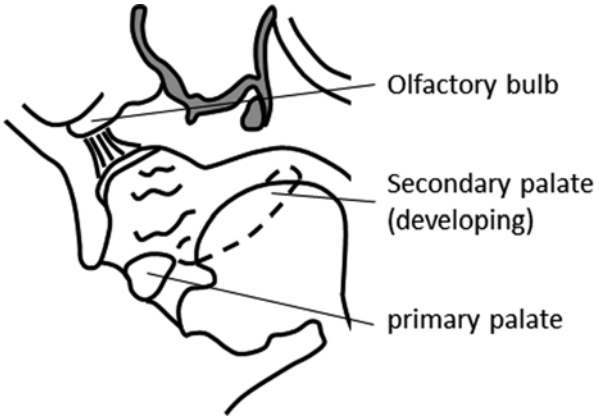

Fig. 7.12 Sagittal section of the embryo in the ninth week

on the roof of the nasal cavity, ectodermal epithelium differentiates into olfactory epithelium. Subsequently, the olfactory epithelium differentiates into olfactory cells and supporting cells, and the olfactory nerve fiber is formed.

7.1.4.2 Ninth to Tenth Week

During the ninth week, the bilateral maxillary prominences form shelf-like growths called lateral palatine processes, which fuse at the midline and produce the secondary palate (Figs. 7.12 and 7.13). The secondary palate fuses with the primary palate, and the formation of the palate is finally completed. The nasal septum fuses with the palate (Figs. 7.14 and 7.15). Ciliated epithelial cells appear in the nasal septum and the inferior nasal conchae, and angiogenesis occurs in lamina propria. Thus, the formation of the fetal face is almost complete by the end of the tenth embryonic week (Fig. 7.16).

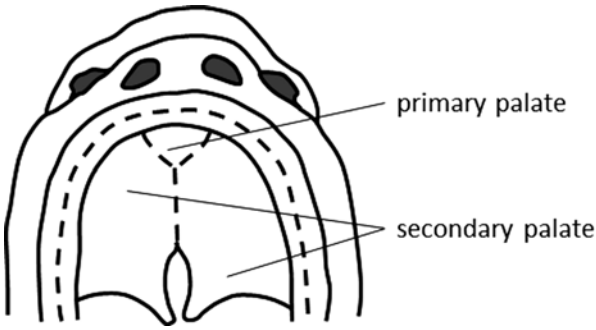

Fig. 7.13 Upward view of palate at the end of tenth week

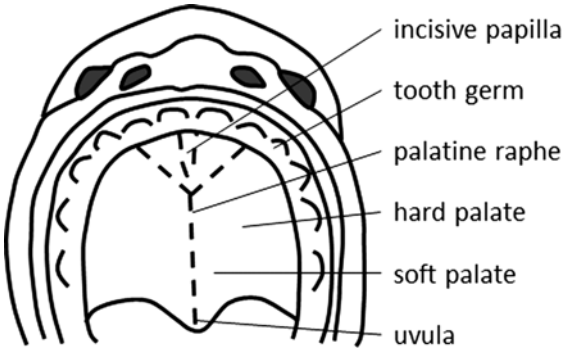

Fig. 7.14 Upward view of palate at the end of the twentieth week

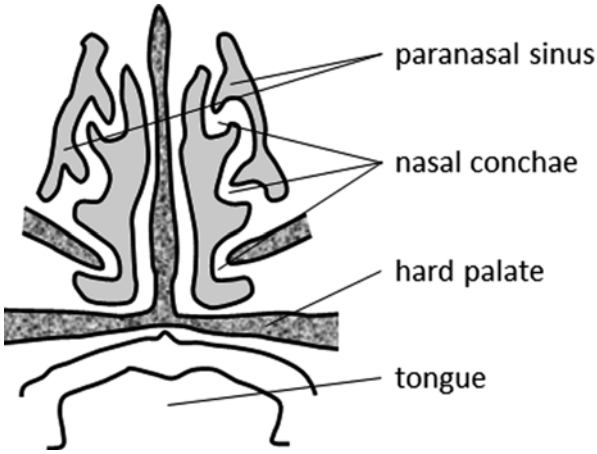

Fig. 7.15 Coronal section of embryo in the twentieth week

7.1.4.3 Eleventh to Fourteenth Week

In the eleventh and twelfth embryonic weeks, the lateral nasal wall, which is anterior to the middle nasal concha, retracts and forms a space known as the ethmoidal infundibulum. The epithelium at the lower part of the ethmoidal infundibulum

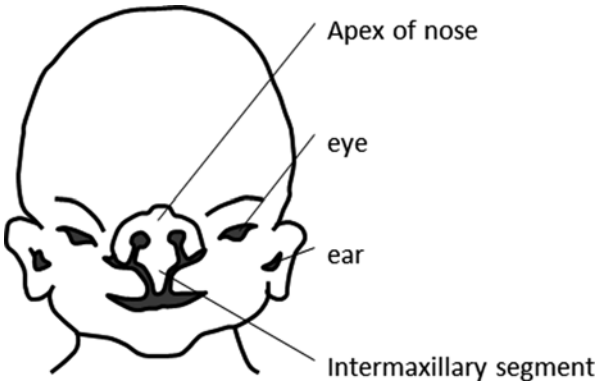

Fig. 7.16 Frontal view of the embryo in the ninth week

migrates toward the maxillary bone and initiates construction of the maxillary sinus. In the thirteenth and fourteenth weeks, the bony structure of the maxilla expands and replaces the cartilaginous tissue of the nasal sac, and the inferior part of the lateral wall is formed. Meanwhile, in the eleventh and twelfth weeks, the process of nasal bone ossification commences and the superior part of the lateral wall is formed. Also, posterior ethmoidal air cells are generated behind the superior nasal meatus. Ossification of the vomer starts in the twelfth week with continued growth until adulthood.

By the thirteenth week, the nasal septum is abundantly covered in ciliary epithelium cells. Goblet cells are present between the ciliary cells, and nasal glands are detectable in the submucosal layer. However, the respiratory epithelial cells of the lateral wall are not yet differentiated, with only a small number of immature cells detectable. Ethmoidal air cells are also covered by undifferentiated respiratory epithelium.

7.1.4.4 Fifteenth to Nineteenth Week

Before the start of the fifteenth week, formation of the superior, middle and inferior nasal conchae is complete, and ossification has started from the inferior nasal conchae. In addition, completion of the nasolacrimal duct occurs in the fifteenth week. In the seventeenth to eighteenth weeks, the anlage of the sphenoid sinus appears at the end of the nasal cavity.

The lining of the nasal cavity contains many ciliated epithelial cells and goblet cells. The lamina propria becomes thicker and more highly vascularized. At the roof of the nasal cavity, formation of the cribriform plate and bundles of olfactory nerve fibers is evident. At this time, all nasal mucosal tissue above the superior nasal concha is olfactory epithelium. At the anterior part of the nasal cavity, the nasal vestibular develops. The border between respiratory epithelial cells, and stratified squamous epithelium is defined, and nasal hair follicles are observed.

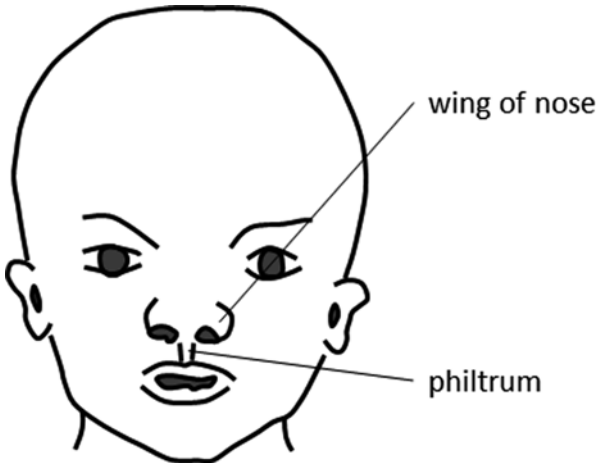

Fig. 7.17 Frontal view of the embryo in the fourteenth week

7.1.4.5 After the Twentieth Week

From the twentieth to the twenty-second week, ossification takes place around the nasolacrimal duct, and the border with the ethmoidal infundibulum becomes clear. Ossification of the nasal conchae rapidly progresses from the lateral wall side. The superior and middle nasal conchae develop as part of the ethmoidal bone, while the inferior nasal conchae form an independent bone. Development of the uncinate process and ethmoidal bulla result in formation of the hiatus semilunaris. Ossification of the maxilla also progresses from the twentieth week onward. Formation of the characteristic structures of the maxillary bone, including the alveolar process, palatine process, inferior orbit wall, zygomatic process, and frontal process, occurs before the twentieth week. Substantial development of the maxillary sinus results in sinus aeration after the twentieth week. Furthermore, ossification of the lateral part of the nasal sac starts and form orbital plate of ethmoid and walls of ethmoidal cells. Histologically, with the exception of the posterior ethmoidal cells, epithelial differentiation of all the paranasal sinuses is complete. The bony structure of the external nose consists of the nasal bone, the frontal process of the maxillary bone, and the nasal part of frontal bone. Ossification of the maxillary bone starts from the canine fossa in the sixth week, extending to the edge of the nasal capsule, and delineating the border zones of the external nose, the lateral wall of the nasal cavity, and the ethmoidal cells. Differentiation of the respiratory epithelium is near completion before the twenty-fourth week.

7.1.4.6 Sixth Month

By the sixth month, the structures of the nose are almost established. After the closure of the external nares by the nasal meatal plug during the eighth embryonic week, the external nares are now recanalized. Formation of the greater alar cartilage and the lateral nasal cartilages are completed; therefore, the appearance of the nose is quite similar to that of an infant (Fig. 7.17).

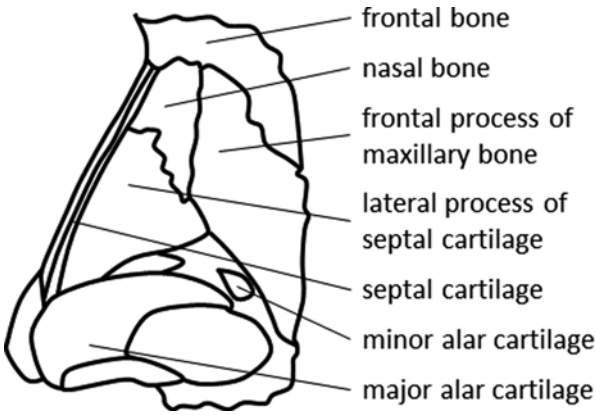

Fig. 7.18 Bony structure of the external nose

7.1.5 Postnatal Growth

7.1.5.1 External Nose

At birth, the lateral and longitudinal diameters of the external nares are 7–8 mm and 5–7 mm, respectively, and they double in size within 6 months. The elliptical shape of each external naris changes from being laterally longer at birth to being longitudinally longer at the time of puberty (Fig. 7.18).

7.1.5.2 Paranasal Sinuses

The maxillary sinus has a lateral diameter of 7–8 mm at birth, and it is located slightly lateral to the inferior nasal concha. Initially, the maxillary sinus expands laterally and reaches the position of the infraorbital nerve within a year. It then expands downward, extending to the middle level of the inferior nasal meatus at 7 years, the level of the nasal base at 12 years, with cessation of growth at 17 years of age.

Ethmoid cellulae are detectable as mucosal invaginations at the sixteenth embryonic week. Each ethmoid cell progressively swells, resulting in a compact structure at 2 years of age. Rapid growth continues until the age of 6 years, and the basic structure of the maxillary sinus is mature at this time point.

Development of the frontal sinus is slower than that of the other sinuses. Aeration is detectable at 1 year of age, extending to the level of superior orbit wall at the age of 8, with growth completed during adolescence. The size of the frontal sinus shows greater interindividual variation compared with the other sinuses.

During the fourth embryonic month, the posterior part of the nasal sac expands and migrates into the sphenoid bone. Aeration increases gradually up to the age of 7 years, and then develops rapidly until adolescence (Figs. 7.19 and 7.20).

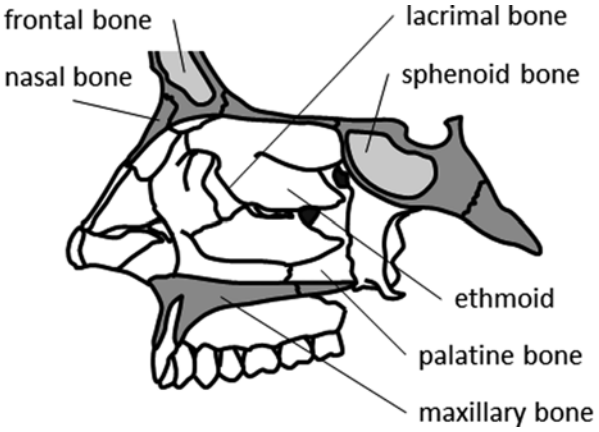

Fig. 7.19 Bony structure of the lateral wall of the nasal cavity

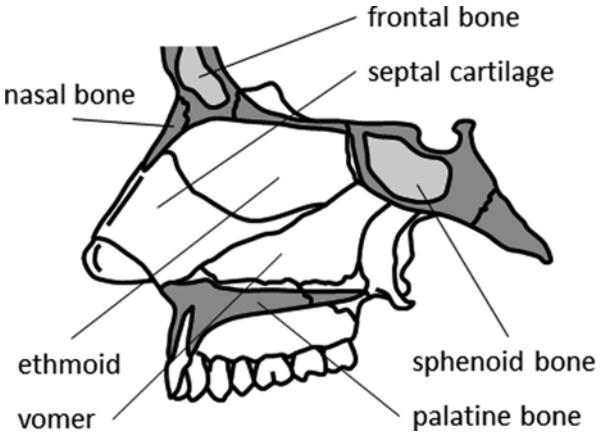

Fig. 7.20 Bony structure of the nasal septum

7.2 Tissue Engineering

7.2.1 Introduction

Because of its central location and morphological variety, the nose is considered to be a very important facial organ for the perception of facial identity. Therefore, when the external nose and its inner components are lost as a result of trauma or cancer surgery, appropriate reconstruction should be performed on the patient. The origin of nasal reconstruction is the Indian (median) forehead flap, which was first conducted in 600 BC. In the sixteenth century, the upper arm flap technique was established [3]. Nasal reconstruction using these autologous flaps requires a donor site and sometimes leaves a severe scar. Another issue is complicated procedure of the grafting. To overcome the disadvantages of autologous flap methods, reconstruction using tissue engineering has been developed. Total nasal reconstruction

requires engineering of the mucosa, cartilage, bone, and skin. In the following chapters, the current status of each of these fields is described.

7.2.2 Mucosal Tissue Engineering

Except for the olfactory and nasal vestibular regions, the entire nasal cavity is covered by respiratory epithelium, which consists of ciliated columnar epithelial cells, goblet cells, and nasal glands. Since the 1980s, epithelial sheets of oral mucosa cells and nasal mucosal cells have been engineered on polymer-based scaffolds [4–6]. Collagen-based membranes are suitable scaffolds for the culture of nasal epithelial cells. In contrast, nasal mucosa has a multilayered structure and it remains a challenge to construct full-thickness engineered nasal mucosa.

7.2.3 Cartilage Tissue Engineering

Cartilage is a flexible tissue that exists in many parts of the body, including the joints, ears, nose, and bronchial tubes. Cartilage is classified into three types: elastic, hyaline, and fibrocartilage, according to the proportion of collagen fibers, proteoglycans, and elastin fibers. The predominant type in the external nose is hyaline cartilage. Cartilage has a limited potential for regeneration because of its avascular property [7, 8]. Candidates cell sources for cartilage engineering are mature chondrocytes and mesenchymal stem cells [9, 10]. Issues with using mature chondrocytes include difficulties with isolation and propagation, while for mesenchymal stem cells, there is a risk of teratoma formation [11, 12]. To date, there are no reports of whole cartilaginous nasal construction using tissue engineering. In clinical practice, injectable tissue-engineered autologous cartilage constructs have yielded good results. For example, chondrocytes harvested from auricular cartilage were cultured and injected into the subcutaneous nasal dorsum with a chondroid matrix, and the results were reported to be satisfactory and sustained [13].

7.2.4 Bone Tissue Engineering

Bone tissue engineering has not been applied in clinical practice because of several limitations and challenges. Bone tissue engineering requires a synergistic interaction between biomaterials, cells, and growth factors. Our research group demonstrated regeneration of the cranial bone using collagen-covered calcium alginate or bone-marrow-derived stromal cells. Both animal studies revealed satisfactory results [14, 15].

7.2.5 Skin Tissue Engineering

The median forehead flap is the most common method for reconstruction of facial skin defects. As may be expected, this method usually leaves a scar on the facial midline. Therefore, tissue-engineered skin without a scar is required, and it should be designed to match the patient's skin texture and color. The cells used in skin engineering are keratinocytes and fibroblasts. In the 1980s, differentiated multilayer sheets of cultured epithelial autografts were used in burn patients [16, 17]. Since then, allogenic skin substitutes have become a mainstream method for concealing skin defects [18]. They prevent the loss of cytokines, growth factors, and other proteins via effusions, and protect from bacterial contamination. Drawbacks of allogenic skin substitutes are their high cost, poor vascularity, and difficulty in constructing the dermal and epidermal layers.

7.3 Regeneration of Olfactory Function

7.3.1 Introduction

Restoration of olfactory function is a primary factor when considering regeneration of nose. Olfactory sensation has not been thought to be as serious as other types of sensory dysfunction such as visual or auditory sensations. Indeed, the study of olfactory sensation has tended to fall behind that of the other senses. The loss of olfactory sensation has a negative influence on quality of life, and, furthermore, the inability to sense harmful substances, for example, gases or rotten foods, may be life threatening. In this context, research on olfactory sensation has recently been recognized as an important topic. This field was highlighted following the award of the Nobel Prize to Buck and Axel in 2004 [19].

7.3.2 Olfactory Dysfunction

Clinically, olfactory dysfunction is classified into three groups: (1) respiratory olfactory dysfunction, which originates in the nasal cavity; (2) peripheral olfactory dysfunction, which is caused by disorders of the olfactory epithelium and/or olfactory nerve; and (3) central nervous system olfactory dysfunction (Fig. 7.21). Respiratory olfactory dysfunction is most commonly observed, accounting for approximately half of all cases of olfactory dysfunction. Sinusitis is the most frequent cause of respiratory olfactory dysfunction, and sense of smell can be improved by performing endoscopic sinus surgery.

Peripheral olfactory dysfunction is typically associated with changes in the olfactory epithelium after a common cold, while nervous system olfactory

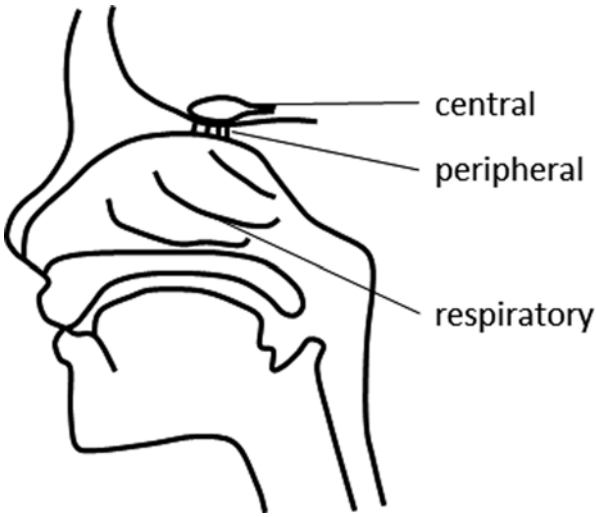

Fig. 7.21 Lesions of olfactory dysfunction

dysfunction is most frequently caused by head trauma. Steroids represent the main treatment for olfactory dysfunction attributed to upper respiratory inflammation. In addition, Japanese herbal medicine has recently been used as a treatment, and approximately 60% of the patients regain olfactory function after the application. Similar treatment is recommended for olfactory dysfunction after head trauma, but the prognosis is worse (approximately 20%) than that for olfactory dysfunction after upper respiratory inflammation [20]. The olfactory nerve is unusual because it has the ability to regenerate. Recently, there have been some new attempts to harness this property.

7.3.3 Structure of Olfactory Mucosa

The olfactory mucosa has a dual-layer structure composed of: (1) the olfactory epithelium containing the body of the olfactory neuron, and (2) the lamina propria containing the olfactory nerve. The olfactory epithelium is characterized by a multicolumnar structure and it has three representative types of cells: sustentacular cells, basal cells, and olfactory neurons (Fig. 7.22). These cell types undergo continuous regeneration throughout life. A subset of the basal cells acts as progenitors for sustentacular cells and olfactory neurons. Olfactory cells are bipolar, with a dendrite that extends to the surface of epithelium, and an axon fiber that grows toward the olfactory bulb. The end of each dendrite is covered with cilia and expresses olfactory receptors [21]. In humans, there are approximately 400 types of olfactory receptors, while rodents have approximately 1,100. Olfactory stimulation is converted into electrical signals that are sent to the olfactory bulb. The life span of olfactory neurons is approximately 2 months in mice. The sheath cells that

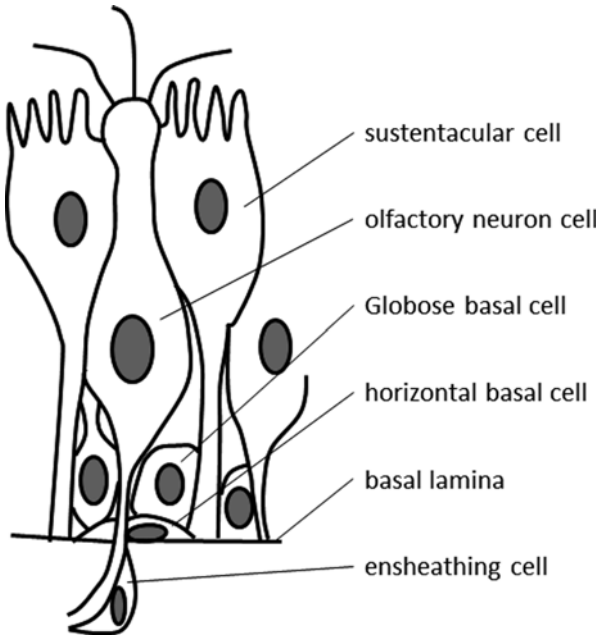

Fig. 7.22 Epithelial cells of the nose

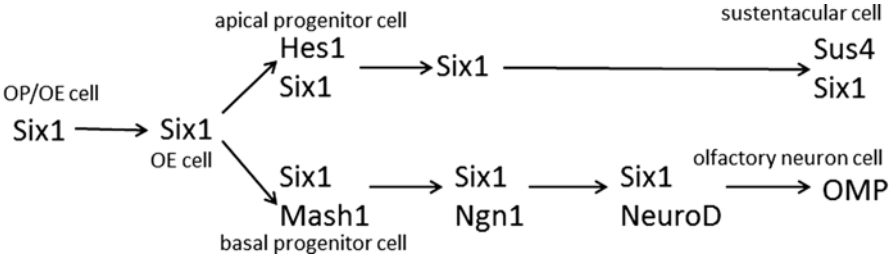

Fig. 7.23 Generation and differentiation of olfactory neurons

surround olfactory nerves have been proposed to be a potential treatment for spinal cord injury [22].

7.3.4 Differentiation of Olfactory Neurons

The genetic cascade that regulates the generation and differentiation of olfactory neurons has been partly elucidated in mice (Fig. 7.23). The first stages are referred to as "early neurogenesis" and the later stages as "established neurogenesis." First, the processes underlying established neurogenesis are explained. The multicolumnar structure of the mature olfactory epithelium is observed after E12.5 in mice.

Olfactory neurons differentiate via this mechanism after E12.5 as well as in response to damage to the olfactory epithelium. In the first step, basal cells, which are the stem cells of the olfactory epithelium, differentiate into basal progenitor cells and apical progenitor cells. At this stage, basal progenitor cells are fated to become sustentacular cells, and apical progenitor cells are fated to become olfactory neuron cells. The former express HES1 and differentiate into mature sustentacular cells that express SUS4. The latter express Mash1, followed by Ngn1, with a gradual increase in NeuroD, and eventually, OMP, a marker of mature olfactory neurons [23–26]. OMP is exclusively expressed in mature olfactory neurons, and is widely used for their identification [27]. In addition, Six1 is expressed on the upstream of them. This fact indicates that Six1 is necessary for a normal olfactory epithelium.

Early neurogenesis occurs prior to establishment of the typical multicolumnar structure. During this stage, unique types of cells known as "pioneer cells" are observed. These cells migrate from the olfactory epithelium to the olfactory bulb and it is suspected that this is related to axon guidance. Six1 plays a key role in the early stages of neurogenesis [24].

7.3.5 Treatment of Olfactory Dysfunction

As described earlier in this chapter, olfactory neurons have the ability to regenerate. Therefore, olfactory dysfunction automatically recovers to some extent even in case of no treatment. In cases of olfactory dysfunction after upper respiratory inflammation, the spontaneous recovery rate is approximately 30%. The primary ongoing treatment is administration of steroid drugs via the nasal cavity [28], which is expected to have an anti-inflammatory effect. In Asia, herbal medications have recently been applied, with some improvements in the prognosis of patients. The beneficial effects are thought to be associated with changes in expression levels of nerve growth factor (NGF) [29].

7.3.6 Future Prospects

Currently, novel findings have been reported from experimental studies. Certain growth factors, for example, NGF and basic fibroblast growth factor (bFGF), are protective to olfactory neurons [30, 31]. In other studies, the antibiotic minocycline is effective for the prevention of apoptosis [32]. Also, anti-IL-6 receptor drugs, administered to inhibit inflammation, have shown a degree of efficacy [33].

Some research groups are testing surgical methods. One approach is transplanting olfactory epithelial cells into the olfactory bulb. Notably, successful transplantation of olfactory epithelium tissue to the olfactory bulb and cerebral cortex has been documented [34, 35].

Cell and tissue cultivation represents another challenge. When considering transplantation or surgical treatment, functional olfactory epithelium needs to be identified or generated. In this respect, cultivation may be necessary. Some studies suggest that it is possible to obtain pluripotent cells from the olfactory epithelium and produce differentiated cells [36]. In the field of olfactory system research, the use of multipotent progenitors such as induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) is yet to be explored.

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Part IV
Development and Regeneration of the Oral Cavity and the Pharynx

Chapter 8
Development and Regeneration of the Oral Cavity and the Pharynx

Nao Hiwatashi and Satoshi Ohno

Abstract The oral cavity and pharynx are anatomical spaces defined by soft and hard tissue structures. These structures act as an aerodigestive tract, and the shape of these spaces changes during speech, swallowing, and respiration. Surgical resection of tumors in oropharynx causes tissue defects and various problems, such as disturbance of mastication, deglutition, and articulation, leading to a decreased quality of life. Thus, there is a need for a reconstructive or regenerative approach to restore lost tissues and prevent postoperative complications. In oropharynx, the reconstructive approach is a mainstay of treating surgical defects so far, and there are few references in the literature regarding the regenerative approach. The reconstructive approach using free flaps has advantages in immediate covering of tissue defects without xenobiotic rejection. However, there exist problems such as the stretching ability in free flaps due to the difference of tissue characteristics. Thus, the regenerative approach should also be evolving in oropharynx.

Keywords Oral cavity • Pharynx • Regenerative medicine • TGFβ • Mesenchymal stem cell

8.1 Anatomy and Development

8.1.1 Anatomy

The oral cavity and pharynx are anatomical spaces defined by soft and hard tissue structures. These structures act as an aerodigestive tract and the shape of these spaces changes during speech, swallowing, and respiration [1]. The oral cavity contains the anterior two-thirds of the tongue, the soft palate, the buccal mucosa, the lips, the floor of the mouth, and the mandible and maxillae. The oral cavity is

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continuous with the oropharynx and is separated from it by a ring of structures including the soft palate, the anterior tonsillar pillars, and the circumvallate papillae. The boundaries of the oral cavity include the hard palate (maxillae) above, the buccal and gingival mucosa laterally, the anterior tonsillar pillars and circumvallate papillae posteriorly, and the mucosa covering the mylohyoid muscles inferiorly.

The pharynx extends from the oral cavity and the nasal choanae anteriorly, the soft palate or velum and portions of the skull base superiorly, the posterior tongue inferiorly, and the pharyngeal constrictor muscles posteriorly. The oropharynx is the central structure of the upper aerodigestive tract. Its upper portion is the nasopharynx, which communicates with the nasal cavity, and its inferior portion is the hypopharynx, which adjoins and surrounds the larynx. The oropharyngeal boundaries are the soft palate superiorly, the tonsillar pillars laterally, the pharyngeal constrictor muscles posteriorly, and the hypopharynx inferiorly. Its contents include the posterior one-third of the tongue, the palatine tonsils, the soft palate, the upper pharyngeal constrictor muscles, and the oropharyngeal mucosa. Thus, the tongue is located in both the oral cavity and the oropharynx. The tonsils are also found in different regions and include the pharyngeal tonsil (adenoid) in the roof of the nasopharynx and the palatine tonsils and lingual tonsils in the oropharynx [2–4].

One classic approach to understanding the anatomy of the oral cavity and pharynx is based on understanding the embryological development of the oral cavity and pharynx. There are many excellent textbooks describing the embryology of these regions in detail [5–7]. The following paragraphs cite and summarize these resources, focusing on the pharyngeal arches, pharyngeal pouches, and pharyngeal clefts.

8.1.2 Embryology of Oral Cavity and Pharynx

Mesenchyme for formation of the structures of the oral cavity and pharynx is derived from paraxial and lateral plate mesoderm, neural crest, and thickened regions of ectoderm known as ectodermal placodes. Neural crest cells originate in the neuroectoderm of forebrain, midbrain, and hindbrain regions and migrate ventrally into the pharyngeal arches. Neural crest cells form the entire viscerocranium (face), parts of the intramembranous bone and cartilaginous regions of the neurocranium (skull). In addition, neural crest cells contribute to other tissue types including dentin, tendon, ligaments, dermis, pia and arachnoid, sensory neurons, and glandular connective tissue.

The pharyngeal apparatus consists of pharyngeal arches, pharyngeal pouches, pharyngeal clefts, and pharyngeal membranes. Although development of these components resembles formation of gills in fishes and amphibians, real gills are never formed in the human embryo.

8.1.2.1 Pharyngeal Arches

Pharyngeal arches begin to develop at day 22 (the beginning of the fourth week) as neural crest cells migrate into the future head and neck regions. Each arch consists of an external covering of ectodermal tissue, a middle core of mesodermal tissue,

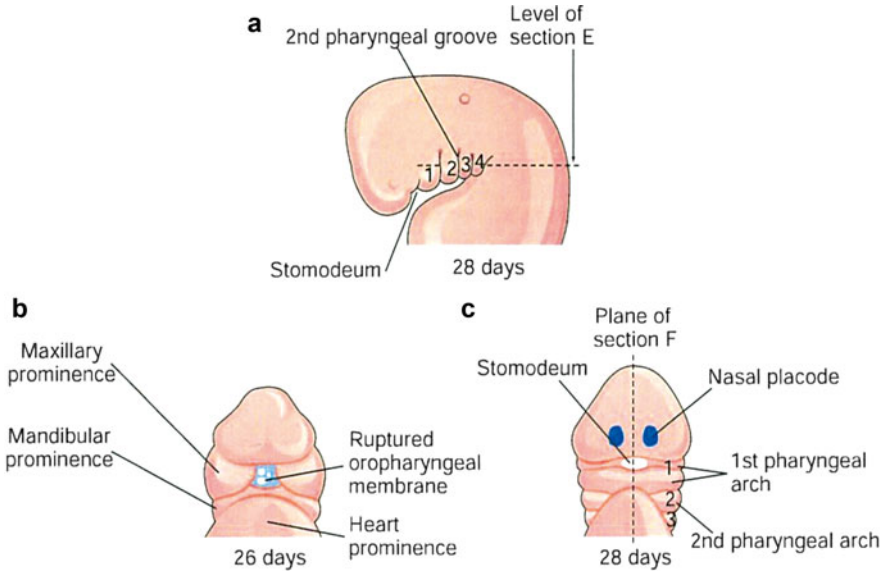

Fig. 8.1 (**a**) Lateral views showing later development of the pharyngeal arches. (**b**, **c**) Ventral or facial views showing the relationship of the first pharyngeal arch to the stomodeum (Cited from Sadler [5])

and an inner lining of endoderm. The first pair of pharyngeal arches appears as surface elevations lateral to the developing pharynx, and the following four arches appear on each side of the future head and neck regions (Fig. 8.1a). The fifth and sixth arches are rudimentary and are not found on the surface. Pharyngeal arches are separated by external clefts and internal pouches.

The first pharyngeal arch plays an important role in formation of the face, the palate, and the superior structures of the oral cavity. Very early during development, the first arch separates into two prominences: the maxillary prominence and the mandibular prominence (Fig. 8.1b). By the end of the fourth week of development, these prominences form four distinct swellings, two maxillary and two mandibular, and are associated with a superior, midline swelling called the frontonasal process. The depression of the surface ectoderm surrounded by these five structures becomes the mouth or stomodeum. The stomodeum is separated from the primordial pharynx by a bilaminar membrane, the oropharyngeal membrane (Fig. 8.1b). At about day 26, the oropharyngeal membrane ruptures to allow the primordial pharynx and foregut to communicate with the amniotic cavity (Fig. 8.1c).

8.1.2.2 Fate of Pharyngeal Arches

The pharyngeal arches contribute extensively to formation of the face, nasal cavities, mouth, larynx, pharynx, and neck. A typical pharyngeal arch contains the following:

- An aortic arch
- A cartilaginous rod that may contribute to formation of the skeleton

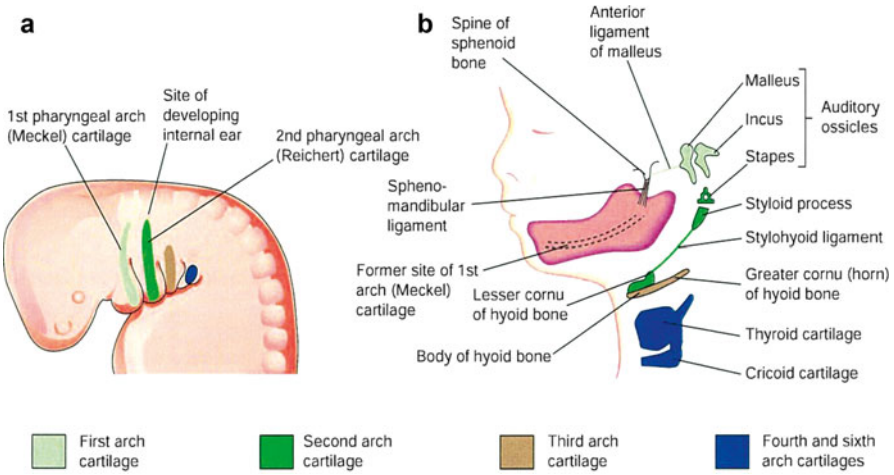

Fig. 8.2 (**a**) The location of the cartilages in the pharyngeal arches. Lateral view of the head, neck, and thoracic regions of a 4-week embryo. (**b**) Similar view of a 24-week fetus illustrating the adult derivatives of the arch cartilage (Cited from Moore et al. [6])

- A muscular component
- A nerve – innervates all structures derived from the specific pharyngeal arch

The derivatives of pharyngeal arch cartilages are mandible and plate as described above. The horseshoe-shaped primordium of the mandible is formed from ventral portions of the first arch. The cartilage disappears as the mandible develops around the cartilage of the first arch by intramembranous ossification (Fig. 8.2). Other derivatives of the first arch cartilage include the malleus and incus from the dorsal end of the first arch cartilage (Meckel cartilage; Fig. 8.2b) and surrounding ligaments. The remaining arches contribute to provide the supporting skeletal and cartilaginous structures, including the stapes in the middle ear, styloid process and part of hyoid bone from second arch, the remainder of hyoid bone from third arch, laryngeal cartilage from the fourth and sixth arch cartilages (Table 8.1).

The musculature derivatives of the first pharyngeal arch are the muscles of mastication and other muscles (Fig. 8.3; Table 8.1). The stapedius muscle, stylohyoid muscle, posterior belly of the digastric muscle, auricular muscles, and muscles of facial expression are derived from the second pharyngeal arch. The stylopharyngeus muscle is derived from the third pharyngeal arch; the cricothyroid, levetor veli palatine, and constrictor muscles of the pharynx are derived from the fourth pharyngeal arch; and the intrinsic muscles of the larynx come from the sixth pharyngeal arch.

A specific cranial nerve (CN) supplies each arch. The dermis and mucous membranes of the head and neck are related to the mesenchyme from the pharyngeal arches, so that these areas are supplied with special visceral afferent nerves. The trigeminal nerve (CN V) is the principal sensory nerve of the head and neck, and its caudal two branches (maxillary and mandibular) innervate the teeth and mucous

Table 8.1 Structures derived from pharyngeal arch components

Arch	Nerve	Muscles	Skeletal Structures	Ligaments
First (mandibular)	Trigeminal (CN V)	Muscles of mastication Mylohyoid and anterior belly of digastric Tensor tympani Tensor veli palatini	Malleus Incus	Anterior ligament of malleus Sphenomandibular ligament
Second (hyoid)	Facial (CN VII)	Muscles of facial expression Stapedius Stylohyoid Posterior belly of digastrics	Stapes Styloid process Lesser cornu of hyoid bone Upper part of body of hyoid bone	Stylohyoid ligament
Third	Glossopharyngeal (CN IX)	Stylopharyngeus	Greater cornu of hyoid bone Lower part of body of hyoid bone	
Fourth and sixth	Superior laryngeal branch of vagus (CN X) Recurrent laryngeal branch of vagus (CN X)	Cricothyroid Levator veli palatine Constrictors of pharynx Intrinsic muscles of larynx Striated muscles of esophagus	Thyroid cartilage Cricoid cartilage Arytenoid cartilage Corniculate cartilage Cuneiform cartilage	

membranes of the palate, mouth, and tongue (Fig. 8.4). The motor nerve of CN V innervates the muscles of mastication. The second, third, and caudal (fourth to sixth) arches are supplied by the facial nerve (CN VII), the glossopharyngeal nerve (CN IX), and the vagus nerve (CN X), respectively. The second to sixth pharyngeal arches innervate the mucous membranes of the tongue, pharynx, and larynx, respectively.

The spaces between the arches are clefts externally and pouches internally. Only the first pair of clefts persist as the external acoustic meatus, while the other clefts are obliterated. The expanding first pouch becomes the tympanic cavity and auditory or Eustachian tube. The remainder contributes to glandular tissue of the head and neck, including the palatine tonsillar crypts from the second pouch, the inferior parathyroid glands and thymus from the third pouch, and the superior parathyroid gland from the fourth pouch (Table 8.1). These tissues will migrate to their adult locations.

The tongue is a complex structure consisting of mucosa, muscles, and both general and special sensory innervation. The anterior two-thirds of tongue is formed

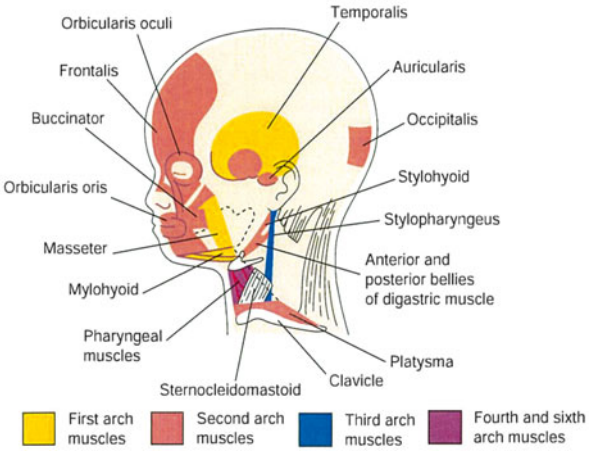

Fig. 8.3 Lateral view of a 20-week fetus showing the muscles derived from the pharyngeal arches. Myoblasts from the second arch migrate from the neck to the head, then form the muscles of facial expression (Cited from Sadler [5])

from mesoderm of the first pharyngeal arch, whereas second to fourth arch mesoderm contributes to the posterior one-third of the tongue, which descends into the oropharynx by 4 years of age. These two tongue regions are separated by a V-shaped cleft called the terminal sulcus. Most of the tongue muscles are derived from myoblasts that migrate from the occipital myotomes, and are innervated by the hypoglossal nerve (CN XII).

8.1.2.3 Molecular Regulation of Mesenchyme Development

Neural crest cells develop from neuroepithelial cells of the surface ectoderm along the edges of the neural plate. Bone morphogenetic protein (BMP) signaling is important in establishing this edge region and then regulates *WNT1* expression, which drives neural crest cells to undergo an epithelial–mesenchymal transition and begin migration into the surrounding mesenchyme. Some cranial neural crest cells originate from rhombomeres, which consists of eight segments in the hindbrain, and migrate into specific pharyngeal arches. There are three main streams of neural crest cell migration from rhombomeres: R1 and R2 migrate to the first arch along with crest cells from the caudal midbrain region; R4 migrate to second arc; and R6 and R7 migrate to arches 4 to 6 (Fig. 8.5). These three distinct streams of neural crest cells provide axonal guidance cues for axons from ganglia forming in the head and neck region. Axons from the trigeminal ganglion enter the hindbrain at R2, while those from the geniculate and vestibuloacoustic ganglia enter at R4 and those from the petrosal and nodose ganglia enter at R6 and R7. No axons project to R3 and R5.

Neural crest cells populating the pharyngeal arches form the skeletal components characteristic of each arch and this process is controlled by pharyngeal pouch endoderm. As pouches form, they express a very characteristic pattern of genes (Fig. 8.6). *BMP7* is expressed in the posterior endoderm, *FGF8* is expressed in the anterior endoderm, *PAX1* expression is restricted to the dorsal-most endoderm of each pouch, and

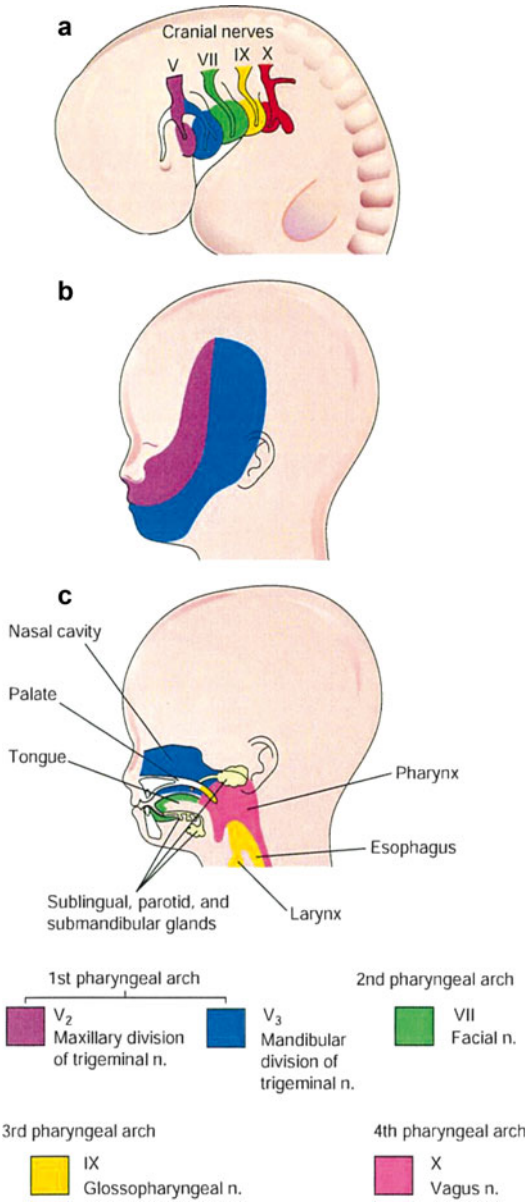

Fig. 8.4 (**a**) Lateral view of a 4-week embryo showing innervation by the cranial nerves to the pharyngeal arches. (**b**) Two caudal branches of the first arch nerve (CN V) distribution of a 20-week fetus. (**c**) The deep distribution of sensory fibers of the nerves to the teeth and mucosa of the tongue, pharynx, nasal cavity, palate, and larynx (Cited from Sadler [5])

SHH is expressed in the posterior endoderm of the second and third pouches. These expression patterns regulate differentiation and patterning of pharyngeal arch mesenchyme into specific structures. In addition, there are different expression patterns of transcription factors in each mesenchyme and they dictate the fate of development. As for the first arch, *HOX* genes are not expressed, but OTX2, a homeodomain-containing transcription factor that is also expressed in midbrain, is expressed. The second

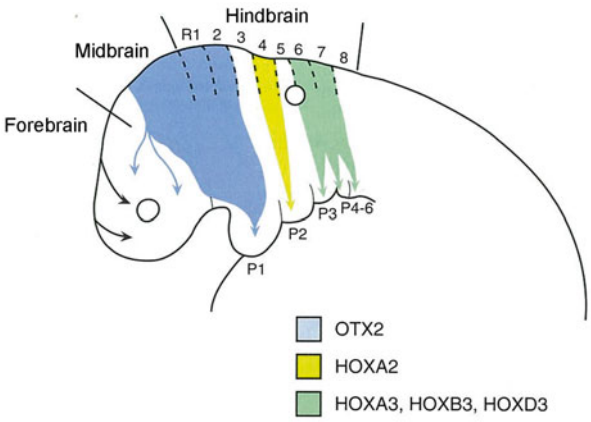

Fig. 8.5 The three streams of neural crest cell migration from the cranial folds into the face and pharyngeal arches. They provide guidance cues for cranial nerve axon outgrowth (Cited from Sadler [5])

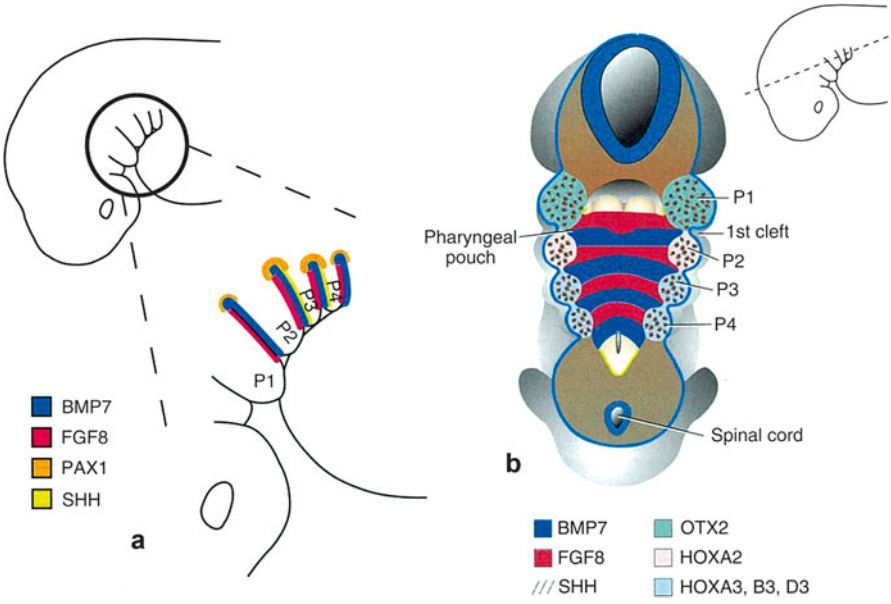

Fig. 8.6 (**a**) The gene expression patterns in pharyngeal arch endoderm and mesenchyme. Endoderm is responsible for patterning the skeletal derivatives of the arches, but the response of the mesenchyme to these signals is dictated by the genes that the mesenchyme express. (**b**) Mesenchyme expression patterns. Neural crest cells from the rhombomeres migrate into the arches and establish the patterns (Cited from Sadler [5])

arch shows *HOXA2* expression, while arches 3 to 6 express members of the third paralogous group of *HOX* genes, *HOXA3*, *HOXB3*, and *HOXD3* (Fig. 8.6b) [5]. With the influence of such diversity of transcription factors, each arch receives signals emanated from pouch endoderm and forms arch-specific structures.

8.2 Regeneration

Surgical resection of tumors causes tissue defects following head and neck surgery. In oropharynx, wide defects of tissue cause various problems, such as disturbance of mastication, deglutition, and articulation, leading to a decreased quality of life. Thus, there is a need for a reconstructive or regenerative approach to restore lost tissues and prevent postoperative complications. In oropharynx, the reconstructive approach is a mainstay of treating surgical defects so far, and there are few references in the literature regarding the regenerative approach. The reconstructive approach using free flaps has advantages in immediate covering of tissue defects without xenobiotic rejection [8]. However, there exist problems such as the stretching ability in free flaps due to the difference of tissue characteristics. Thus, the regenerative approach should also be evolving in oropharynx.

Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward tissue regeneration [9]. Three elements have been adopted for tissue regeneration: regulatory factors, cells, and scaffolds. Tissue regeneration will be achieved when these three elements are appropriately provided. At present, there are some reports regarding each element.

Regulatory factors modulate cell behavior such as extracellular matrix production, cell proliferation and differentiation. Growth factors are peptide molecules produced by various cells and representative regulatory factors.

Transforming growth factor (TGF) β3 is one of the TGFβ isoforms that activate nuclear-translocating Smads and other intercellular proteins, leading to altered expression of target genes by first binding to TGFβ type II receptors before pairing together with two type I receptors [10]. Embryonic wounds are known to heal perfectly with no scars [11]. There are far fewer inflammatory cells in embryonic wounds, the inflammatory cells present are less differentiated, and the length of time that inflammatory cells are present is markedly reduced compared with adult wounds [12]. Embryonic wounds express very high levels of TGFβ3 but very low levels of TGFβ1 and TGFβ2 compared to adult wounds [11]. Application of anti-TGFβ1 and anti-TGFβ2 antibodies to healing adult rodent wounds results in markedly reduced scarring without an increase of wound infection [13, 14]. Pan-neutralization of all three TGFβ isoforms does not improve scarring [13]. The oral mucosa heals rapidly with relatively less scarring than skin. TGFβ3 levels are relatively higher in oral mucosa wounds compared with skin wounds [15]. Ohno et al. investigated regenerative and scar preventive effects of TGFβ3 in the rat buccal mucosa [16]. The administration of 0.5 ug/mL TGFβ3 before wounding accelerated reepithelialization and reduced scar formation in buccal mucosa wounds (Fig. 8.7). Histologic examination revealed suppressed inflammatory reactions and the restoration of elastin and hyaluronic acid, which affects viscoelasticity in the tissue and plays a key role in the reduction of tissue contracture (Figs. 8.8 and 8.9) [17]. Anti-scarring effects of TGFβ3 are also reported in vocal folds [18, 19].

Epidermal growth factor promotes the proliferation of keratinocytes isolated from gingival tissue of the rabbit in vitro [20]. When the oral mucosa is injured,

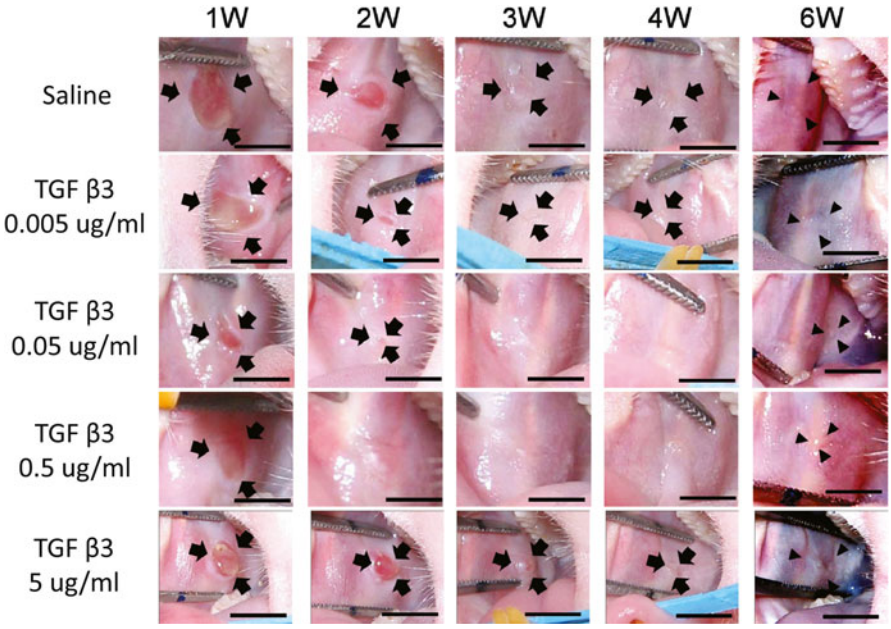

Fig. 8.7 Representative images of wound healing in buccal mucosa over time. TGFβ3 -treated wounds were rapidly re-epithelialized and left less scarring. *Arrows* indicate raw surfaces. *Arrowheads* indicate scars. Bars represent 5 mm (Cited from Ohno et al. [16])

a physiological concentration of EGF in saliva stimulates the proliferation and migration of oral epithelial cells for wound healing [21]. Keratinocyte growth factor (KGF) has been described as a powerful paracrine agent, secreted by stromal fibroblasts, which specifically affects different epithelial cell types, especially keratinocytes [22]. KGF is highly expressed in buccal mucosal fibroblasts compared with dermal fibroblasts [23]. This is one possible reason why oral mucosa wounds heal faster. Blaimauer et al. demonstrated that an appropriate concentration of EGF and KGF was needed for proliferation of keratinocytes in vitro [24]. EGF and KGF were found to be secreted by fibroblasts, not by keratinocytes.

Cells are an integral component of tissues and produce various extracellular matrices such as collagen, elastin, and hyaluronic acid. There are two major strategies to regenerate tissues in cell therapy: one is to supply stem cells that have regenerative effects in wounds, and the other is to transplant differentiated cells in their native form.

Marynka-Kalmani et al. isolated multilineage cells from human oral mucosa lamina propria (hOMSCs: human oral mucosa stem cells) [25]. hOMSCs exist in the cord-like structures within the oral mucosa lamina propria and express Oct4 and Sox2, which are known to maintain the pluripotency of embryonic stem cells (Fig. 8.10). hOMSCs were found to produce several growth factors such as fibroblast

growth factor and vascular endothelial growth factor (VEGF). However, implanted hOMSCs formed bilineage mixed tumors in vivo. Thus, further research is needed to apply hOMSCs to cell therapy.

Mesenchymal stem cells (MSCs) are plastic-adherent cells that can be obtained from bone marrow. MSCs have been found to have multilineage potential and

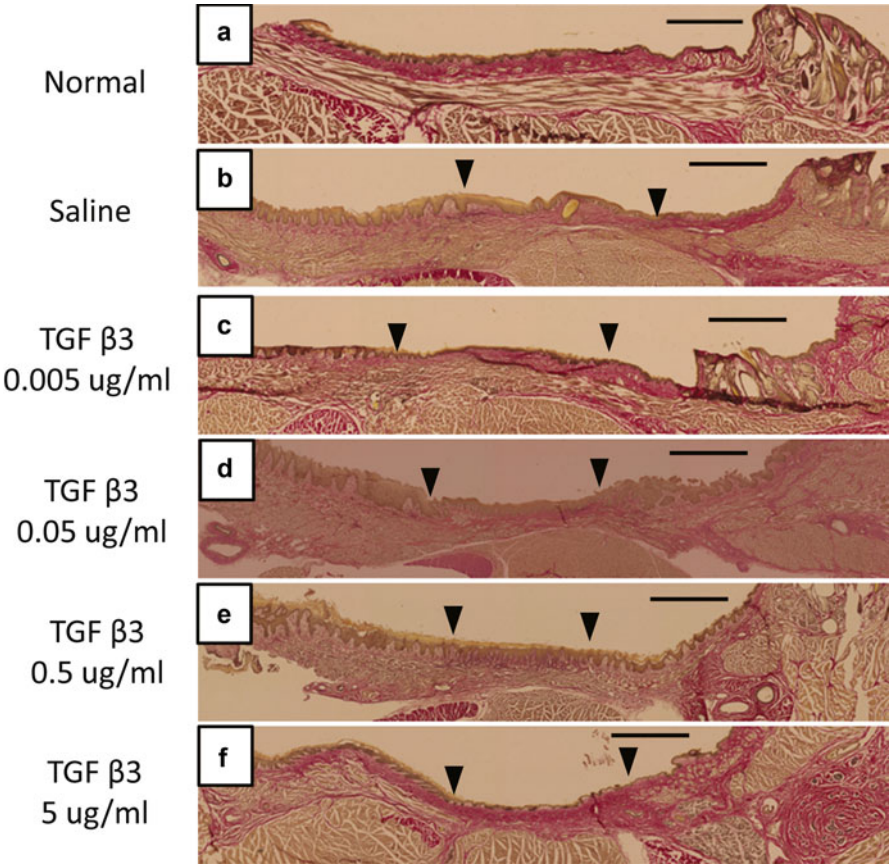

Fig. 8.8 Histological findings of buccal mucosa wounds at 6 weeks postoperatively using Elastica van Gieson staining. Better restoration and less fusion of muscle and scar tissue in the 0.05 and 0.5 ug/mL TGFβ3 groups compared with the saline group and the other two TGFβ3 groups. Collagen deposition was found to be almost the same in all groups, except for the 5 ug/mL TGFβ3 group in which dense collagen deposition was observed. Elastin in the 0.5 ug/mL TGFβ3 group appeared to be well-organized, similar to that of the normal buccal mucosa, whereas it was decreased in the others. (**a**, **g**) Normal buccal tissue. (**b**, **h**) Saline group. (**c**, **i**) TGFβ3: 0.005 ug/mL group. (**d**, **j**) TGFβ3: 0.05 ug/mL group. (**e**, **k**) TGFβ3: 0.5 ug/mL group. (**f**, **l**) TGFβ3: 5 ug/mL. *Arrowheads* indicate the border between normal tissue and scarred tissue. *E* epithelium, *SM* submucosal layer, *M* muscle layer. Bars represent 1 mm (**a**–**f**) and 50 um (**g**–**l**) (Cited from Ohno et al. [16])

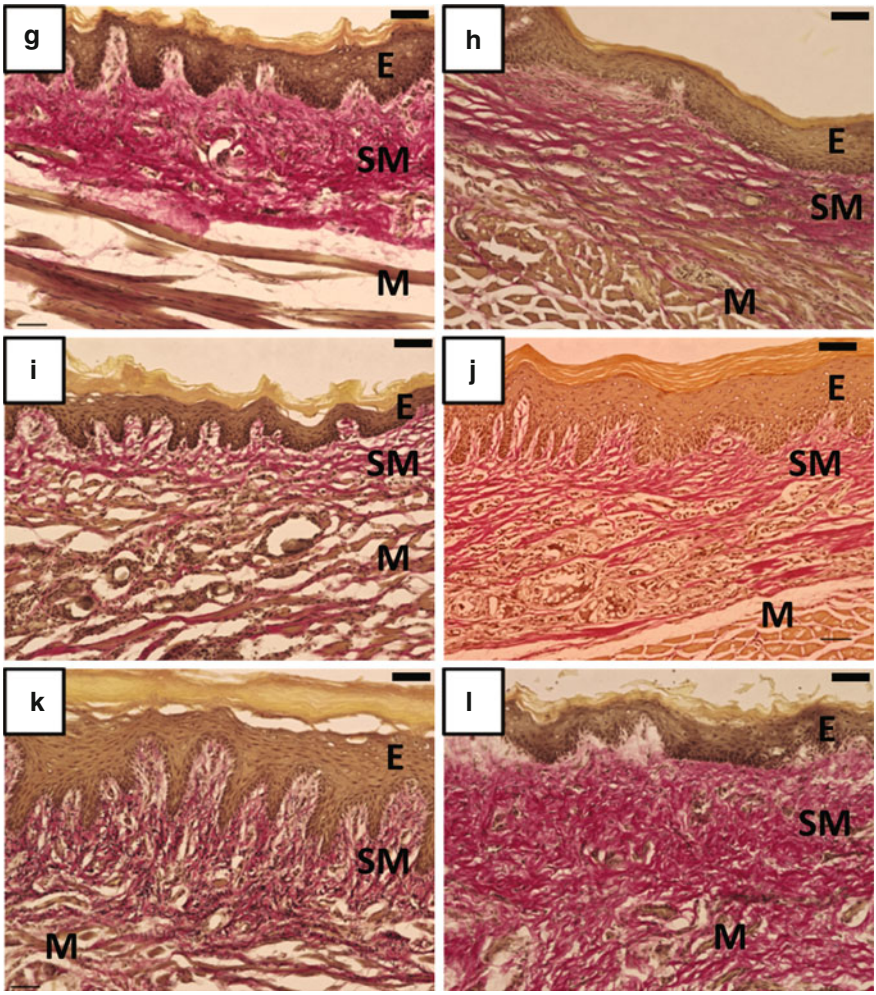

Fig. 8.8 (continued)

produce several growth factors, including VEGF and hepatocyte growth factor (HGF). HGF is a potent mitogen for mature hepatocytes and also has strong antifibrotic effects in various organs [26, 27]. Implantation of MSCs into scarred vocal folds has been shown to increase hyaluronic acid distribution in the lamina propria and ameliorate vocal fold scarring [28, 29]. Adipose derived stem cells (ADSCs) can be easily obtained from the adipose tissue and have a marker expression that is similar to that of MSC [30]. ADSCs are considered to have a regenerative ability similar to MSCs in various organs [31, 32]. Aziz Aly et al. investigated the usefulness of implantation of MSCs and ADSCs for oral mucosa ulcers in dogs. Cells

were injected into oral ulcers 3 days after chemical burn. MSCs and ADSCs showed rapid wound healing, mild subepithelial inflammatory cell infiltration, and higher VEGF DNA concentration in wounds.

Ohki et al. applied cell sheets to human mucosal defects caused by esophageal endoscopic submucosal dissection [33]. Epithelial cells were collected from oral mucosa specimen and cultured. Confluent cell sheets were harvested as a single, unsupported contiguous cell sheet, retaining cell-to-cell junctions as well as deposited extracellular matrix on the basal surface of the sheet. They showed that transplantation of cell sheets prevented stricture of the esophagus.

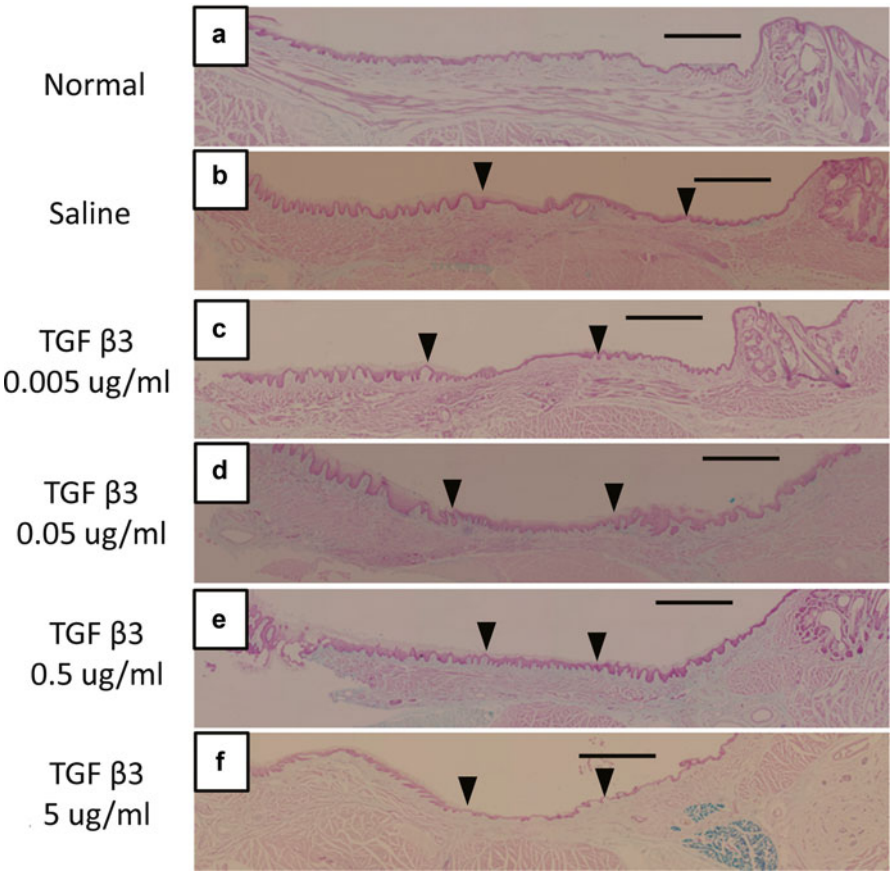

Fig. 8.9 Histological findings of buccal mucosa wounds at 6 weeks postoperatively using Alcian blue staining. Hyaluronic acid appeared to be well-restored in the 0.05 and 0.5 ug/mL TGFβ3 groups, whereas it was less so in the others. (**a**, **g**) Normal buccal tissue. (**b**, **h**) Saline group. (**c**, **i**) TGFβ3: 0.005 ug/mL group. (**d**, **j**) TGFβ3: 0.05 ug/mL group. (**e**, **k**) TGFβ3: 0.5 ug/mL group. (**f**, **l**) TGFβ3: 5 ug/mL. *Arrowheads* indicate the border between normal tissue and scarred tissue. *E* epithelium, *SM* submucosal layer, *M* muscle layer. Bars represent 1 mm (**a**–**f**) and 50 um (**g**–**l**) (Cited from Ohno et al. [16])

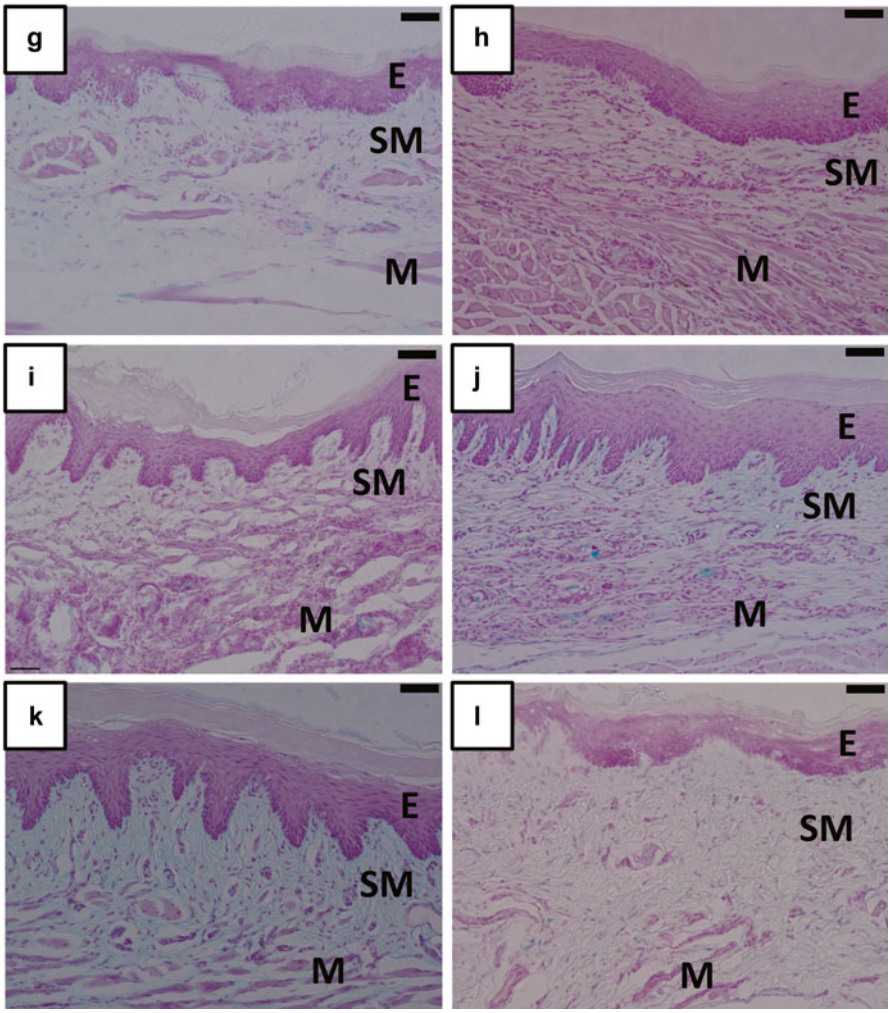

Fig. 8.9 (continued)

Scaffolds replace tissue defects and maintain a space for cells to proliferate and secrete extracellular matrices. Biodegradable materials are ideal for scaffolds because they can be replaced by regenerated tissue after completion of regeneration.

An atelocollagen sponge is reported to be effective as a scaffold. Terudermis (Terumo Co, Tokyo, Japan) is a dehydrothermally cross-linked atelocollagen sponge derived from calf dermis. It is approved for clinical use in the treatment of injured dermis and epidermis by the Ministry of Health in Japan. Strong fibrillar atelocollagen and heat-denatured atelocollagen, which promotes

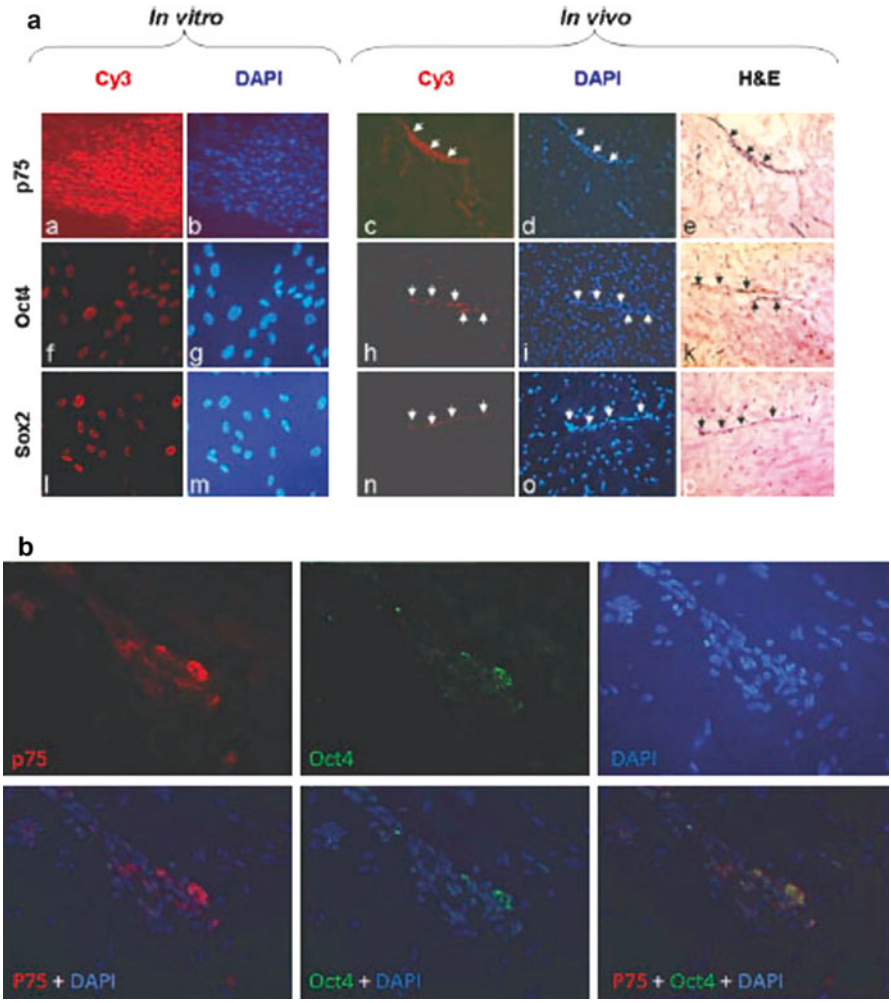

Fig. 8.10 (**a**) In vitro and in vivo expression of primitive stem cell markers in oral mucosa. The majority of hOMSC stain positively for p75, which is a marker of neural crest stem cells (*a*, *b*). Groups of hOMSC stain positively for Oct4 and Sox2 (*f*, *g*, *l*, *m*). Immunostaining of oral mucosa lamina propria (OMLP)-derived sections localizes p75 to cord-like structure of high-cellular density as indicated by DAPI and H&E staining (*arrows* in (*c*–*e*)). Oct4 (*arrows* in (*h*–*k*)) and Sox2 (*arrows* in (*n*–*p*)) are localized to similar structures. (**b**) Colocalization of Oct4 and p75 in a cord-like structure in the OMLP. *Abbreviations*: *DAPI* 4,6-diamidino-2-phenylindole, *H&E* hematoxylin and eosin (Cited from Marynka-Kalmani et al. [25])

excellent cell migration, are mixed at a ratio of 9:1 and lyophilized to create a layer of collagen sponge about 5 mm thick. This sponge has many large pores that permit cell entry (Fig. 8.11), and it is degraded gradually in vivo by endogenous collagenase [34, 35]. Bessho et al. used Terudermis to cover gingival mucosa defects in human [35]. Good epithelialization and almost no

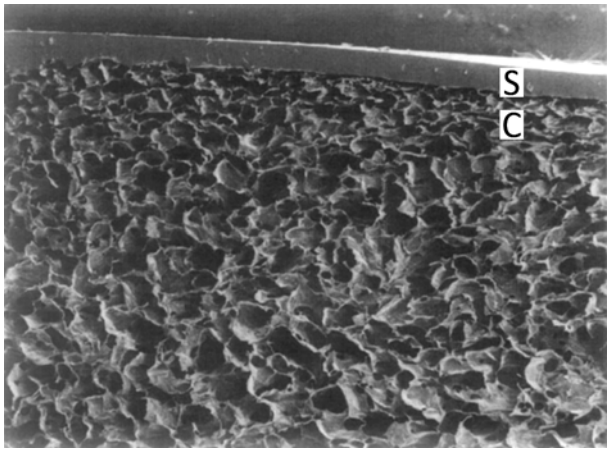

Fig. 8.11 Scanning electron micrograph showing the whole section of Terudermis (Terumo Co, Tokyo, Japan). *S* silicone layer, *C* atelocollagen layer (Cited from Bessho et al. [35])

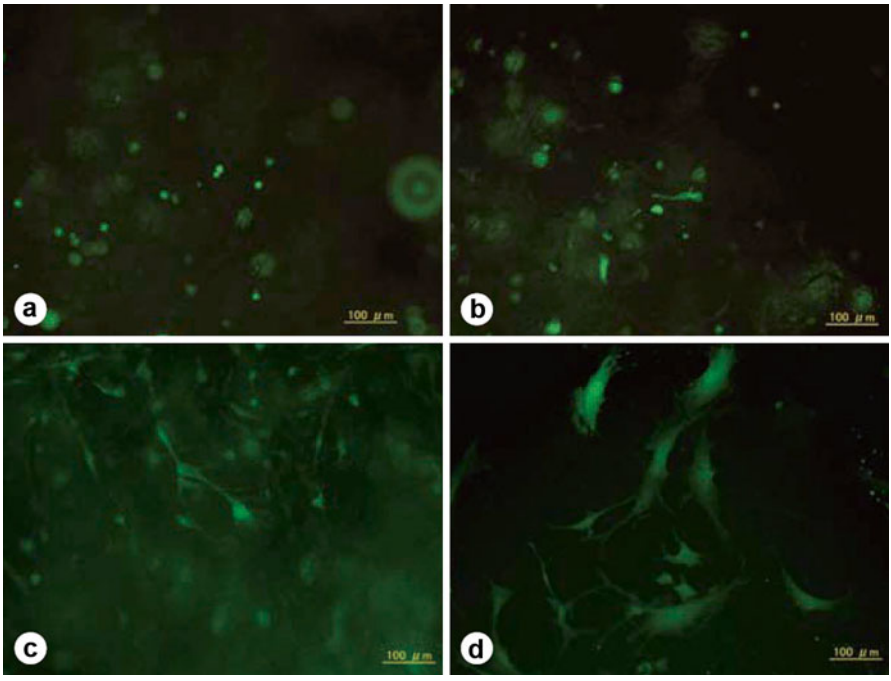

Fig. 8.12 Morphology of green fluorescent protein-labeled MSCs on an atelocollagen sponge in the incubator. Cellular extension was observed from day 1. Intercellular network developed in a time-dependent manner. (**a**) Day 0. (**b**) Day 1. (**c**) Day 3. (**d**) Day 5 (Cited from Ohno et al. [36])

contracture were observed at 1 year postoperatively. MSCs can adhere to an atelocollagen sponge and proliferate in vitro (Fig. 8.12) [36]. Implantation of an atelocollagen sponge with autologous MSCs into scarred vocal folds

significantly increased hyaluronic acid distribution and decreased dense collagen deposition in the lamina propria, leading to better mucosal vibration [28]. Further research using atelocollagen sponges is warranted in oropharynx regeneration.

Yonezawa et al. reported a way to cover mucosal defects with fibrin glue and polyglycolic acid (PGA) sheets (MCFP technique) in the resection of rabbit tongue model [37]. The MCFP technique means that wounds are covered with a biodegradable fabric composed of PGA sheets and fibrin glue. First, a solution of fibrinogen is applied to wounds, and then wounds are covered with PGA sheets slightly smaller than the extent of resection. Finally, a mixture of a solution of fibrinogen and thrombin, which stiffens about 3 min later, is sprayed over wounds. Wound surface-covering agents made of PGA have been clinically [38, 39] and experimentally [40] reported to be effective for wound healing. Compared to primary closure, mucosal defects covered by the MCFP technique showed a delay of wound healing up to 2 weeks postoperatively; however, there was no significant difference in morphological changes at 4 weeks postoperatively.

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Part V
Development and Regeneration of the Larynx

Chapter 9
Laryngeal Development

Yo Kishimoto

Abstract Research examining the origin and the development of the larynx has been performed since the late nineteenth century and has provided valuable insights into the anatomy and pathologies of the larynx. In this chapter, we provide a brief review of laryngeal anatomy, followed by a summary of the prenatal development of the larynx. Finally, we discuss development of the framework, the muscles, and the nerves of the larynx in detail.

A better understanding of the processes of laryngeal development is crucial to understand the anatomy of the larynx and the mechanisms of breathing, swallowing, and voice production.

Keywords Larynx • Development • Human • Embryogenesis

9.1 Anatomy of the Larynx

The larynx connects the lower part of the pharynx to the trachea. It is located in the ventral midline of the neck at C3–C6 vertebral levels, just behind the strap muscles [1]. The larynx functions primarily as a sphincter. It also acts as a conduit for air flow, an organ to produce sounds, and to protect the lower respiratory tract from aspiration [2]. The larynx is composed of laryngeal cartilages, ligaments, muscles, and membranes, over which a mucosal lining is draped. Laryngeal actions such as swallowing, respiration, and phonation are driven by the intrinsic and extrinsic laryngeal muscles.

The laryngeal cartilaginous framework consists of the thyroid, cricoid, and epiglottis, as well as the paired arytenoid, corniculate, and cuneiform cartilages (Fig. 9.1a, b). These cartilages are connected by ligaments, forming two joints called the cricoarytenoid joint and the cricothyroid joint. The thyroid cartilage is the largest laryngeal cartilage and consists of two laminae meeting anteriorly at an

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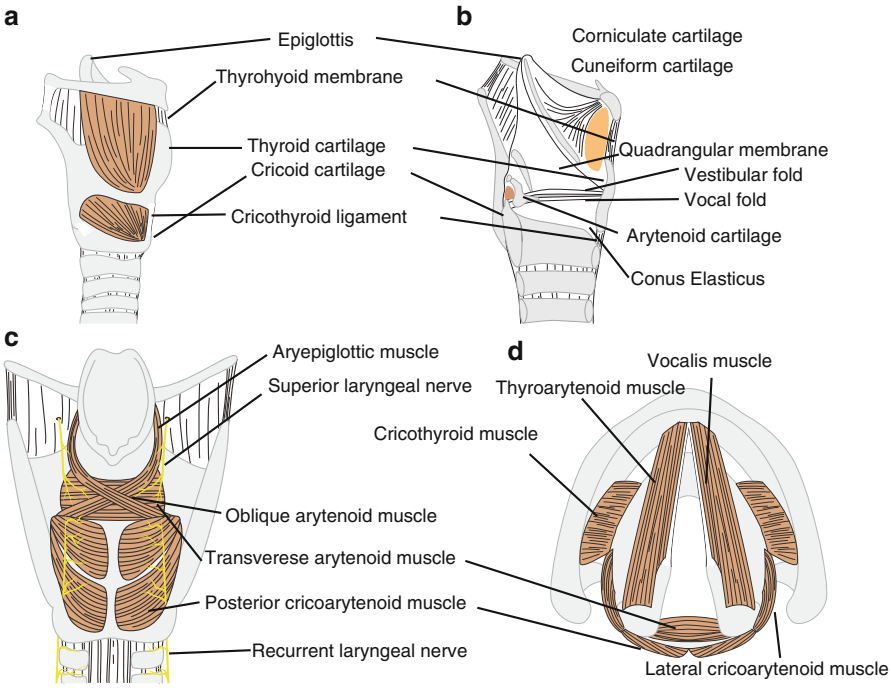

Fig. 9.1 Anatomy of the larynx. Lateral view (**a**), sagittal view (**b**), posterior view (**c**), and superior view (**d**)

approximately 90° angle [1]. The angle shows gender differences with that of male being smaller than that of female. The sharper angle of the male thyroid cartilages makes the cartilages appear to protrude at the C4 vertebral level (Adam's Apple) compared to that of females. Two pairs of cornua extend from the posterior surface of this cartilage. The superior cornua are attached to the hyoid bone by the thyrohyoid membrane and the inferior cornua articulate with the cricoid cartilage [1]. The oblique line of the thyroid cartilage is found on the body of each plate and serves as the attachment of the sternothyroid and thyrohyoid muscles.

The cricoid cartilage is the only complete ring-like cartilage in the larynx. It is located at the level of the transverse process of the C6 vertebrate and has a wider posterior surface (lamina) compared to its anterior arch. This cartilage has a pair of cricoarytenoid joints on the upper surface of the posterior lamina and is supported by posterior cricoarytenoid ligaments [1]. The arytenoid cartilages are paired, three-sided pyramid-shaped cartilages that articulate with the cricoid cartilages. The arytenoid cartilage has an anterior vocal process for attachment of the vocal ligament and a lateral muscular process. The arytenoid cartilage pivots and slides at the cricoarytenoid joint, which allows for adduction and abduction of the vocal process.

The epiglottis is a teardrop-shaped, thin plate of elastic fibrocartilage expanding superiorly and attaches to the laryngeal prominence and the hyoid by the thyroepiglottic

ligament and hyoepiglottic ligament, respectively. Laterally, the epiglottis is attached to the arytenoid cartilages by the aryepiglottic folds. This structure provides the epiglottis a degree of flexibility to bend posteriorly during swallowing.

The ligaments and membranes of the larynx include the conus elasticus (cricovocal membrane), the vocal ligament, the cricothyroid ligament/membrane, the thyrohyoid membrane, the hyoepiglottic ligament, the thyroepiglottic ligament, the quadrangular membrane, and the vestibular ligament (Fig. 9.1a, b). These structures serve as the support for the cartilages and function as anatomical barriers in the larynx.

The cricovocal membrane is the anterior portion of the cricothyroid membrane. It attaches to the vocal process of the arytenoid cartilage posteriorly and to the angle of the thyroid cartilage anteriorly. At the superior boundary, the membrane is thickened between these two points and is known as the vocal ligament, which is covered by the vocal fold (true vocal cord). The quadrangular membrane and the vestibular ligament form the aryepiglottic fold and the vestibular fold, respectively. The paired vestibular folds are referred to as the false vocal cords [3], and the ventricles are located between the vestibular folds and the vocal folds.

The muscles of the larynx are categorized as extrinsic or intrinsic. The extrinsic laryngeal muscles have an attachment outside of the larynx. They are responsible for movements of the larynx, and are divided into elevators and depressors. The thyrohyoid, palatopharyngeus, stylopharyngeus, and inferior pharyngeal constrictor muscles elevate the larynx, while the omohyoid, sternohyoid, and sternothyroid muscles depress the larynx. The intrinsic laryngeal muscles attach between laryngeal cartilages, and include the thyroepiglottic muscle, the aryepiglottic muscle, the lateral/posterior cricoarytenoid muscle, the transverse/oblique arytenoid muscle, the vocalis muscle, the thyroarytenoid muscle, and the cricothyroid muscle (Fig. 9.1c, d). Of these muscles, only the posterior cricoarytenoid muscles open the glottis, while all remaining muscles that attach to the arytenoid cartilage close the glottis. The cricothyroid muscles tense the vocal cords and the vocalis muscle attunes the vocal cords finely by relaxing specific portions of the cords [4].

The larynx is innervated by the superior and recurrent laryngeal branches of the vagus nerve. The superior laryngeal nerve divides into an internal and external branch. The internal branch of the superior laryngeal nerve enters the larynx running through the thyrohyoid membrane with the superior laryngeal artery. This branch carries sensory fibers to the larynx above the vocal cords, autonomic fibers to the laryngeal glands, and taste fibers to the epiglottis and adjacent root of the tongue [1]. The external branch of the superior laryngeal nerve runs with the superior thyroid artery. They separate at the superior pole of the thyroid, and the external branch of the nerve passes anteriorly and inferiorly to innervate the cricothyroid muscle, a portion of the inferior pharyngeal constrictor muscle, and the cricopharyngeus muscle. The recurrent laryngeal nerve enters the larynx with a branch of the inferior thyroid artery called the inferior laryngeal artery (Fig. 9.1c). All or the intrinsic laryngeal muscles, except for the cricothyroid muscle, are innervated by motor and sensory fibers of the recurrent laryngeal nerve. The sensory fibers provide general sensation to the subglottic area [1].

9.2 Laryngeal Development Overview

Laryngeal development has been studied for over a century, and different concepts have developed concerning the developmental processes. However, new technologies, including computer-generated 3D reconstructions of the human embryo or organized human embryonic collections, allow researchers to reexamine embryologic events, the origin of laryngeal components, and their anatomic relationships over time [2]. In this section, we review laryngeal development during the embryonic period.

The laryngotracheal groove, the respiratory primordium, appears as an outgrowth in the floor of the caudal end of the pharyngeal foregut during the 4th week of human gestation (Fig. 9.2a). Initially, this primordium of the tracheobronchial tree is separated from the hepatic primordium by the septum transversum, and is observed as a longitudinal fissure caudal to the fourth pair of pharyngeal pouches (Fig. 9.3a). By the end of the 4th week, the laryngotracheal groove has deepened to form a pouch-like laryngotracheal diverticulum located ventral to the caudal portion of the foregut. This respiratory diverticulum is separated from the pharyngeal floor by the primitive laryngopharynx. The primitive pharyngeal floor and the primitive laryngopharynx eventually develop into the infraglottic and the supraglottic region of the adult larynx, respectively [5]. With elongation of the diverticulum, it becomes surrounded by splanchnic mesenchyme (primordial embryonic connective tissue), and, during the 5th week, its distal end enlarges to form a bronchopulmonary bud that eventually develops into the lower respiratory tract. Later in the 5th week, the respiratory diverticulum migrates superiorly, and its cranial portion develops as the infraglottis [5]. The pulmonary epithelium and glands of the larynx, trachea, and bronchi develop from the endoderm lining the laryngotracheal groove. The connective tissue, cartilaginous components, and smooth muscle of the lower respiratory system are derived from the splanchnic mesoderm surrounding the laryngotracheal tube [6].

Initially, the laryngotracheal diverticulum communicates with the foregut. With the descending outgrowth of the diverticulum, it becomes separated from the primordial pharynx by the development of tracheoesophageal ridges. The ridges gradually approach each other and form the *tracheoesophageal* septum by the end of the 5th week. This septum divides the cranial portion of the foregut into ventral and dorsal regions. The ventral region is the laryngotracheal tube that eventually becomes the larynx, trachea, bronchi, and lungs, and the dorsal region is the primordium of the oropharynx and esophagus [6]. The respiratory primordium maintains communication with the pharynx via an opening of the laryngotracheal tube into the pharynx called the laryngeal orifice [7].

The laryngeal orifice becomes the primitive glottis during the 5th week (Fig. 9.2b). During this week, the mesenchyme at the cranial end of the laryngotracheal tube proliferates rapidly and forms the paired arytenoid swellings at the same anatomic level as the fourth pharyngeal pouch. Initially, this primitive glottis is shown to be a slit-like aperture, it becomes narrow as the arytenoids swellings grow towards the tongue. Further, there is a prominence at the ventral ends of

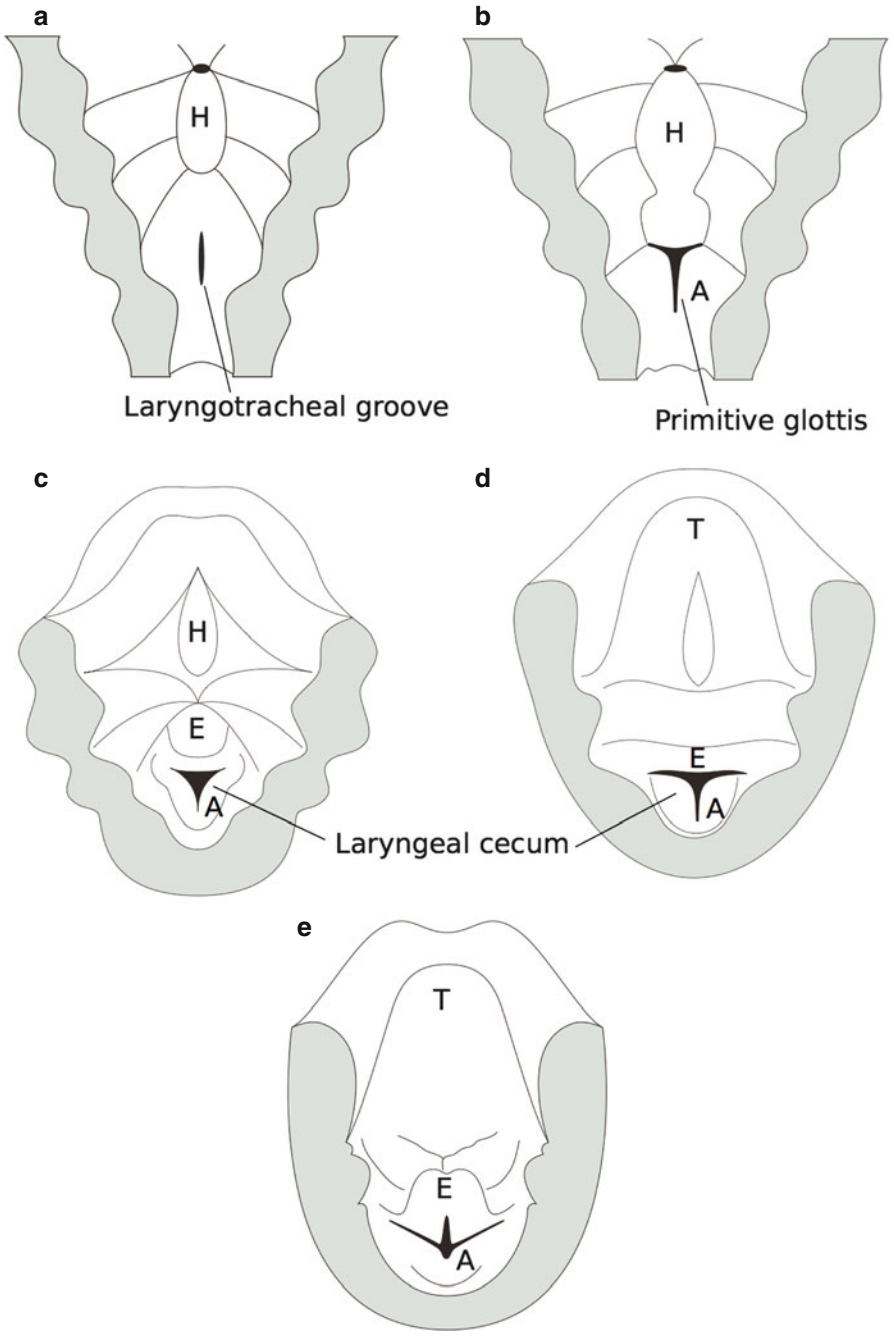

Fig. 9.2 Stages in the development of the larynx. Week 4 (**a**), week 5 (**b**), later in week 5 (**c**), later in the 6th to 7th week (**d**), and fetal period (**e**), *A* arytenoid swelling, *H* hypobranchial eminence, *E* epiglottis, *T* tongue (Reproduced from Ferlito [5] and Moore et al. [6])

the third and fourth pharyngeal arches, the hypobranchial eminence, that develops caudal to the copula with the proliferation of mesenchyme. The caudal part of this eminence develops as the epiglottis by the 10th week, and the rostral part forms the pharyngeal portion of the tongue. At the beginning of the 6th week, the primitive

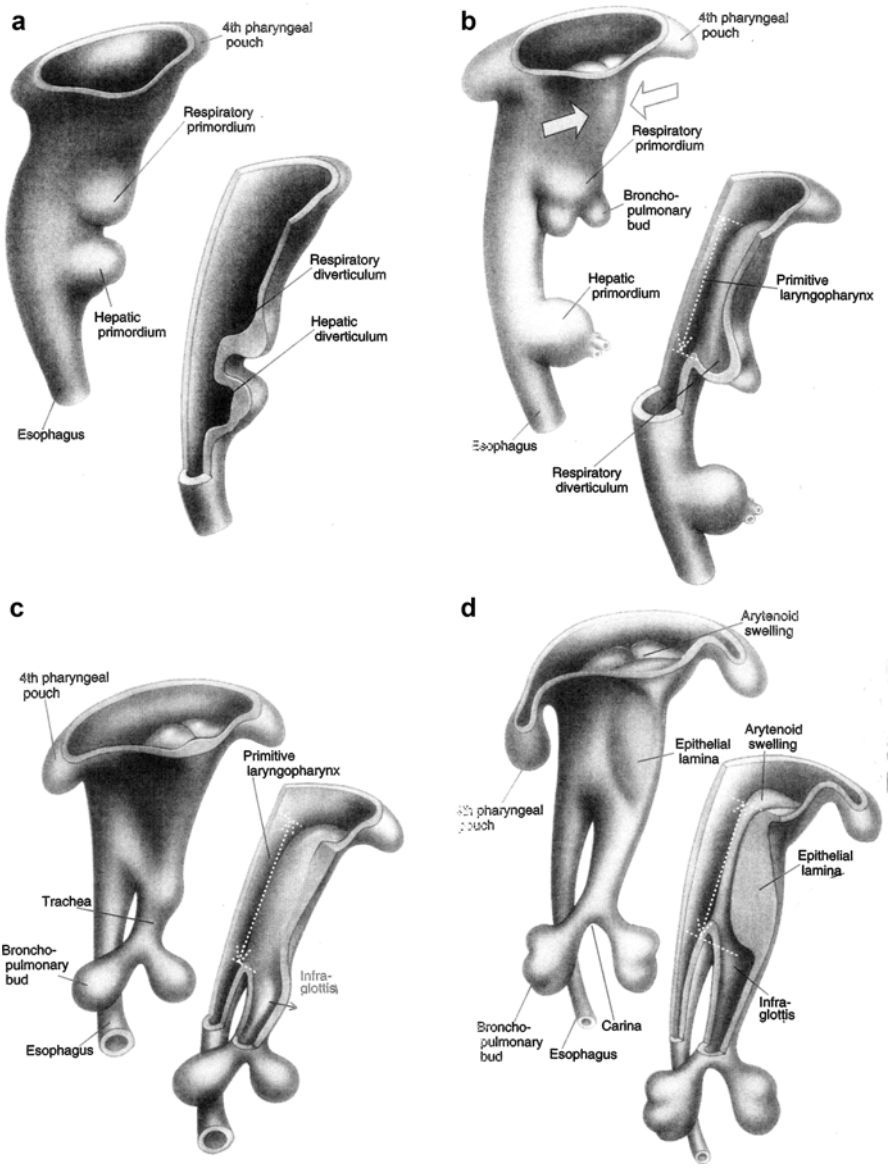

Fig. 9.3 Laryngeal development at Carnegie stage 11 (**a**), stage 12 (**b**), stage 13/14 (**c**), stage 15 (**d**), stage 16 (**e**), stage 17/18 (**f**), stage 19/23 (**g**), and fetal period (**h**) (Reproduced from Ferlito [5])

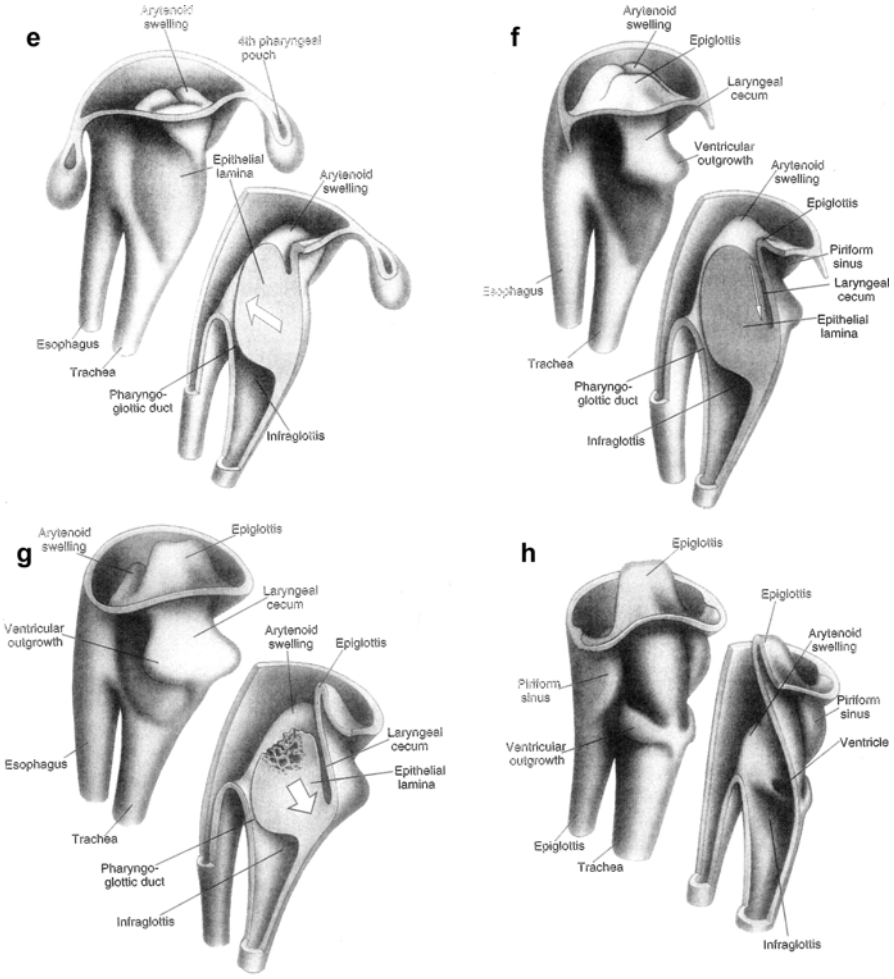

Fig. 9.3 (continued)

glottis surrounded by the hypobranchial eminence and arytenoid swellings forms a T-shaped laryngeal aditus that reduces the laryngeal lumen to a narrow slit with the development of the epithelial lamina, a condensation of mesenchymal tissue outlining the hyoid, thyroid, and cricoid bodies (Fig. 9.3d) [5, 8]. Later in the 6th week, the primitive glottis is obliterated completely except for the pharyngoglottic duct, and the laryngeal cecum begins to develop between the arytenoid swellings and the epiglottis, forming the vestibular outgrowth (Figs. 9.3d and 9.4e) [9]. The laryngeal cecum descends and reaches to the level of the glottic region during the 7th week (Fig. 9.4f, g) [5].

During the 8th week, the epithelial lamina begins to recanalize in a dorso-cephalic to ventro-caudal direction, resulting in a communication between the ventral laryngeal

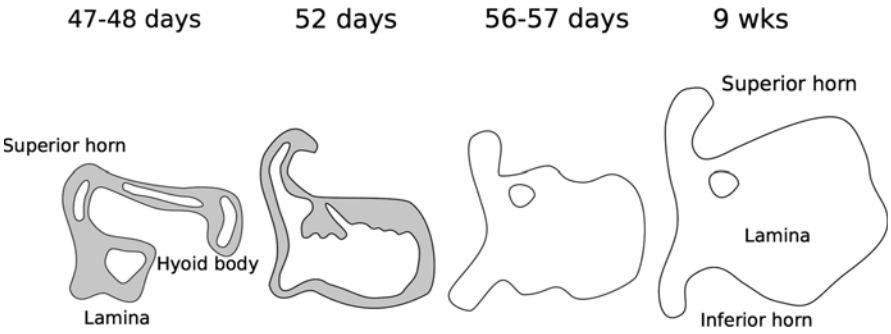

Fig. 9.4 Chondrification of the thyroid cartilage (Reproduced from Zaw-Tun and Burdi [28])

cecum and the dorsal pharyngoglottic duct. Complete recanalization is necessary for the communication between the supraglottis and the infraglottis, and incomplete recanalization may lead to supraglottic and glottic webs and atresia [5]. Recanalization of the laryngeal lumen is completed by the 10th week and creates a pair of lateral recesses called the laryngeal ventricles (Figs. 9.3e and 9.4h). The ventricles are bounded by the vocal folds and ventricular folds, which appear as outgrowths from the pharyngeal floor related to the third pharyngeal pouches at the end of the 7th week. During 8th to 9th week, they modify their morphology as laryngeal framework enlarges [10]. At the 8th week, the epithelium of the larynx is pseudostratified columnar and showing a clear basement membrane [11].

9.3 Development of the Laryngeal Framework

The laryngeal framework includes the thyroid, cricoid, and epiglottic cartilages and the paired arytenoid, corniculate, and cuneiform cartilages. These cartilages begin to chondrify at the beginning of the 8th week [12]. The thyroid, cricoid, and arytenoid cartilages are composed of hyaline cartilage and may exhibit calcification during the young adult ages. However, the other cartilages are composed of fibroelastic cartilage with little tendency of ossification over time.

Each pharyngeal arch has cartilaginous component, and the fourth and sixth arch cartilages fuse to form the thyroid, cricoid, arytenoid, corniculate, and cuneiform cartilages, but not the epiglottis. The thyroid cartilage develops from the ventral ends of the cartilages of the fourth pharyngeal arch. Initially, the rudiment of this cartilage appears as two plates in front of the fourth pouch and cleft, and lies between the constrictors at the end of the 1st month. Bilateral small foci of cells appear in the laminae of the thyroid cartilage at the 7th week indicating the subsequent chondrocyte differentiation (Fig. 9.4). A week later, they joined cranially and caudally [9, 13], and complete confluence of these foci and formation of the definitive cartilages occurs postnatally [14]. Bilateral centers for the cricoid arch and the arytenoids appear at almost same time as those for the thyroid cartilage.

The thyroid cartilage is composed of two laminae, and they join the hyoid bone at the upper edges. The superior horns of the thyroid cartilage are a direct prolongation of the greater horns of the hyoid. By the end of the 2nd trimester (21st week), the hyoid and the thyroid horns become separated by the thyrohyoid membrane, and the thyroid cartilage shows a neonatal form [13]. If there is a failure in this disconnection process, a total ossified hyothyroideal element or triticeous cartilage will be observed [8]. Occasionally, a foramen thyroideum appears near the posterior border of the thyroid lamina, below the superior thyroid tubercle. It represents a failure of the complete chondrification of mesenchymal elements derived from the fourth and sixth arches, and is associated with the presence of neurovascular elements. If it is not invaded with neurovascular elements, the foramen chondrifies completely and disappears [15].

The cricoid cartilage develops from the sixth pharyngeal arch, and the cricoid condensations appear at the end of the 6th week [9]. At the 8th week, the cricoid is bilateral, located dorsally, and it grows rapidly by the end of this week [16]. The cricoid cartilage changes its growth pattern from interstitial to perichondrial during the fetal period. Chondrification of the cricoid begins from two centers. Initially, they unite ventrally and extend chondrification from the lateral into the dorsal plate [17]. The chondrification of the cricoid cartilage is thought to occur from inferior to superior; however, the process has not been clarified [8]. This circular cartilage is not distensible, becomes the most narrow part of the subglottic airway, and remains similar in shape throughout postnatal development [18].

The arytenoid, corniculate, and cuneiform cartilages are derived from the sixth arch cartilages. The early condensation of the arytenoid, which seems to be larger than that of the cricoid, is observed during the 6th week, and the precartilaginous template with vocal, muscular, and apical processes is completed by the beginning of the 8th week [5, 16]. Initially, the arytenoid cartilages join the cricoid cartilages by mesenchymal condensations and the cricoarytenoid joints are undeveloped. The development and chondrification of the arytenoid and corniculate cartilages takes place during the 12th week, and the development of cuneiform cartilages starts at 28 weeks [19, 20]. Laryngeal hyaline cartilages are derived from branchial mesoderm, while elastic cartilages develop from the mesoderm of the floor of the pharynx. While most of the arytenoid is composed of hyaline cartilage, the vocal processes consist of elastic cartilage as they develop in association with the vocal folds. The vocal process is the last portion of the arytenoid to be chondrified completely [5].

As mentioned above, the cartilage of the epiglottis develops from mesenchyme of the hypobranchial eminence from the third and fourth pharyngeal arches [21]. The epiglottis itself can be observed during the 6th to 7th week [9], and the epiglottic cartilage appears as a loose concentration of small cells lying under the lining membrane of the hypobranchial eminence at the end of the 2nd month or the beginning of the 3rd month [16]. By the 21st week, the epiglottis is located at a similar level as the uvula of the soft palate, and the epiglottis and the soft palate appear to overlap between the 23rd to 25th week (Fig. 9.5a) [2]. Chondrification of the epiglottis occurs during the 20th week [19], and the fibro-cartilaginous structure matures during the fetal period [22]. During this period, the lateral wings of the

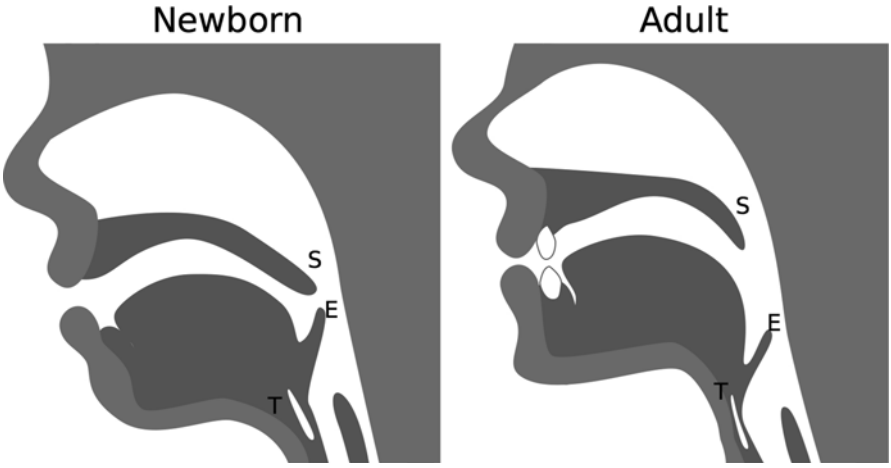

Fig. 9.5 Sagittal sections of the pharyngolarynx at the newborn (**a**) and at adulthood (**b**)

epiglottis and the upper and outer part of the arytenoid swellings elongate, forming the aryepiglottic folds. During the 3 years after birth, the epiglottis grows rapidly and reaches the adult form [23].

During the fetal period, the larynx is located at a higher position in the neck compared to its postnatal position, and the region from the tip of the epiglottis to the lower border of the cricoid cartilages extends between C1 to C3/4 [12]. At birth, the larynx lies between C1 and C4. The high laryngeal positioning in the newborn restricts the range of vocalization, while allowing for the epiglottis to rest posterior to the soft palate, which permits simultaneous sucking and respiration and obligate nasal breathing [24]. During the 3 years after birth, the larynx grows rapidly and descends, with the lower rim of the cricoid cartilages reaching to the C4/5 level. There is little change from this period to puberty, when the larynx approaches the adult position with the growth of the thyroid cartilage (Fig. 9.5b) [12].

The laryngeal cartilages grow in the postnatal period, and begin to exhibit sexual dimorphism towards the end of pubertal growth. The thyroid laminae are larger and the angle between the laminae is more acute in the male. This allows a greater length for the vocal cords in males, resulting in a deeper voice. At puberty, the vocal fold length in males is more than twice that in females [13].

Calcification and ossification of the laryngeal cartilages begins in the young adult after the 2nd decade, but has rarely been reported in infancy. Although the timing of the ossification of the laryngeal cartilages is highly variable and is not related to gender, ossification exhibits a very similar progressive pattern between males and females (Fig. 9.6) [25, 26]. The ossification process of the laryngeal cartilages has been well investigated, and some features have been identified [27]. In the thyroid cartilage, ossification starts at the postero-inferior border of the lamina and extends into the inferior horn and along the posterior border. Subsequently, ossification develops upwards along the posterior border of the lamina towards the

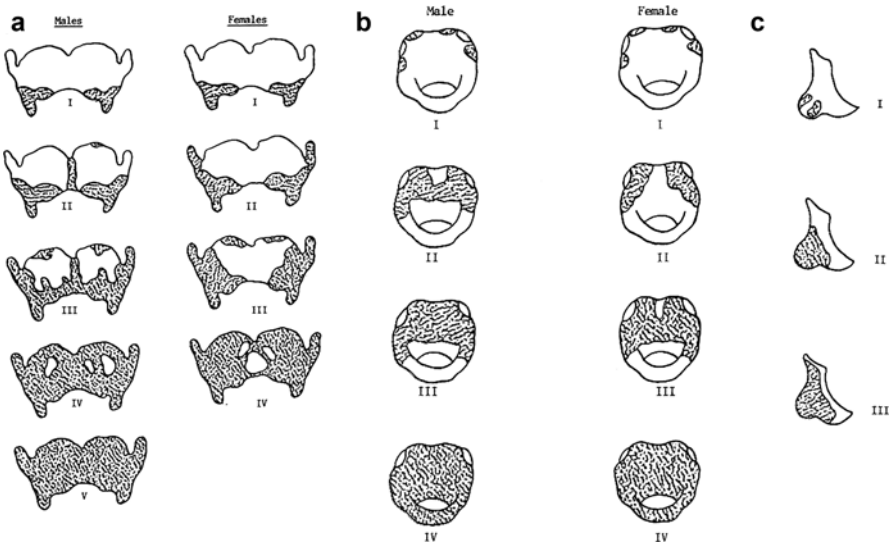

Fig. 9.6 Stages of radiographic opacity of the laryngeal cartilages, thyroid cartilage (**a**), cricoid cartilage (**b**), and arytenoid cartilage (**c**) (Reproduced from De la Grandmaison et al. [25])

superior horn before spreading into the main part of the lamina. The center of the laminae may be ossified or not; however, the entire cartilage may ossify in males, producing an os thyroideum. Variation in ossification of the thyroid cartilage between genders has been reported, and females generally show less ossification compared to males at similar ages [26].

In the cricoid cartilages, ossification starts at the superior edge of the posterior lamina and spreads down the sloping lateral sides to ossify the anterior arch. In the arytenoid cartilages, ossification is common in the main body and muscular process. However, ossification is seen rarely in the vocal process. There is a difference in the distribution of calcification and ossification of the arytenoid cartilage between genders, and the arytenoid cartilage tends to calcify in females and ossify in males [13, 25].

9.4 Development of the Laryngeal Muscle

During development, muscle precursors from paraxial mesoderm migrate ventrally, differentiate into myoblasts, and form the branchial muscles. Muscles of the larynx originate from myoblasts of the fourth and sixth pharyngeal arches [2].

During the 4th week, two planes of circularly disposed cells, which are separated by mesoderm of the fourth arch, appear around the pharynx and larynx. These planes are referred to as the inner and the outer constrictors. The inner constrictor forms the internal intrinsic muscles of the larynx and a part of the pharyngeal musculature. The outer constrictor appears to be a part of the pharyngeal musculature

and cricothyroid muscle [16]. Muscle-specific regulatory genes are expressed prior to myoblast migration; however, the intrinsic laryngeal musculature does not exhibit the signs of muscle organization until after adjacent cartilaginous structures have begun to condense [2].

By the 8th week, mesenchymal condensation takes place, forming the anlage of the intrinsic laryngeal muscles. The differentiation of muscles follows the formation and growth of the laryngeal cartilages, and the formation of the arytenoid cartilage converts the dorsoposterior part of the inner constrictor into the posterior cricoarytenoid muscle, the upper part into the arytenoid muscle, and the ventral part into the thyroarytenoid and lateral cricoarytenoid muscles [16].

In the 6th or 7th week of gestation, the outer constrictor increases in thickness and elongates ventrally, which places it in contact with the cricoids. The muscle fibers of the outer constrictor that are attached to the thyroid cartilage become attached to the cricoid at the end of the 2nd or the beginning of the 3rd month. These fibers form the cricothyroid muscle [16].

The sites of each muscle are recognizable by the 8th week, and, at the 3rd month, muscle fibers with striation are discernible [28, 29]. Since the cricothyroid muscle is derived from the inferior pharyngeal constrictor muscle, which develops from the fourth pharyngeal arch, it is innervated by the superior laryngeal nerve. Meanwhile, the other intrinsic muscles of the larynx are derived from the sixth pharyngeal arch and are innervated by the recurrent laryngeal nerve. Condensations for the posterior cricothyroid, arytenoid, and posterior cricoarytenoid muscles appear at 44 days [30], and the vocalis is reported to differentiate during the 8th week [29]. Muscles of the epiglottis are not observed in the 60 mm embryo, although the cartilage is well developed [16].

9.5 Development of the Laryngeal Nerve

As shown in Table 9.1, structures derived from the fourth and sixth pharyngeal arches are innervated by the vagus nerve (Cranial Nerve X). Motor neurons of the vagus nerve arise from rhombomeres 7 and 8, and the nerve is formed by fusion of the nerves of the fourth and sixth arches (Fig. 9.7) [31]. The vagus nerve has large visceral efferent and visceral afferent components that distribute to the heart, the foregut and its derivatives, and to a large part of the midgut.

The nerve of the fourth arch becomes the superior laryngeal nerve that innervates the cricothyroid muscle and the constrictors of the pharynx. The nerve of the sixth arch becomes the recurrent laryngeal nerve, and distributes to the laryngeal musculature. The superior laryngeal nerve and the recurrent laryngeal nerve can be observed at 33 days and 37 days during gestation, respectively [9].

The course of the recurrent laryngeal nerves is different between left and right. At 6 weeks, the recurrent laryngeal nerves run medially from the vagus nerve to the laryngeal area under the symmetric sixth aortic arches. On the right side, the sixth aortic arch disappears during week 7, and the recurrent laryngeal

Table 9.1 Structures derived from pharyngeal arch components

Arch	Skeletal Structures	Muscles	Nerves	Ligaments
First	Malleus Incus	Muscles of mastication (temporalis, masseter, medial and lateral pterygoids) Mylohyoid and anterior belly of digastric Tensor tympani Tensor veli palatine	V	Anterior ligament of malleus Sphenomandibular ligament
Second	Stapes Styloid process Lesser cornu of hyoid Upper part of body of the hyoid bone	Muscles of facial expressions (buccinator, auricularis, frontalis, platysma, orbicularis oris and oculi) Stapedius Stylohyoid Posterior belly of digastric	VII	Stylohyoid ligament
Third	Greater cornu of hyoid Lower part of body of the hyoid bone	Stylopharyngeus	IX	
Fourth and sixth	Thyroid cartilage Cricoid cartilage Arytenoid cartilage Corniculate cartilage Cuneiform cartilage	Cricothyroid Levator veli palatine Constrictors of pharynx Intrinsic muscles of larynx Striated muscles of the esophagus	X Superior laryngeal branch of vagus Recurrent laryngeal branch of vagus	

nerve moves superiorly to course around the right subclavian artery, a derivative of the fourth aortic arch. Meanwhile, the persisting sixth aortic arch on the left is in a more caudal position relative to the larynx, and the left recurrent laryngeal nerve hooks around the ductus arteriosus, the distal portion of the sixth aortic arch (Fig. 9.8) [32]. At the 8th week, the transitional area between the laryngopharynx and the esophagus is innervated by the recurrent laryngeal nerve. While nerve fibers have not reached the epithelium of the ventricle, some fibers are observed near the vestibule [11]. The number of myelinated fibers in the laryngeal nerves decreases with age [33].

Occasionally, an abnormal involution of the right fourth aortic arch occurs, resulting in the absence of the subclavian artery in its normal position. This mislocation of the artery shifts the recurrent laryngeal nerve to the higher position in the neck. This nonrecurrent laryngeal nerve diverges from the vagus nerve and runs directly into the larynx at the level of the upper half of the thyroid lobe. A rare left nonrecurrent laryngeal nerve is associated with situs inversus [34].

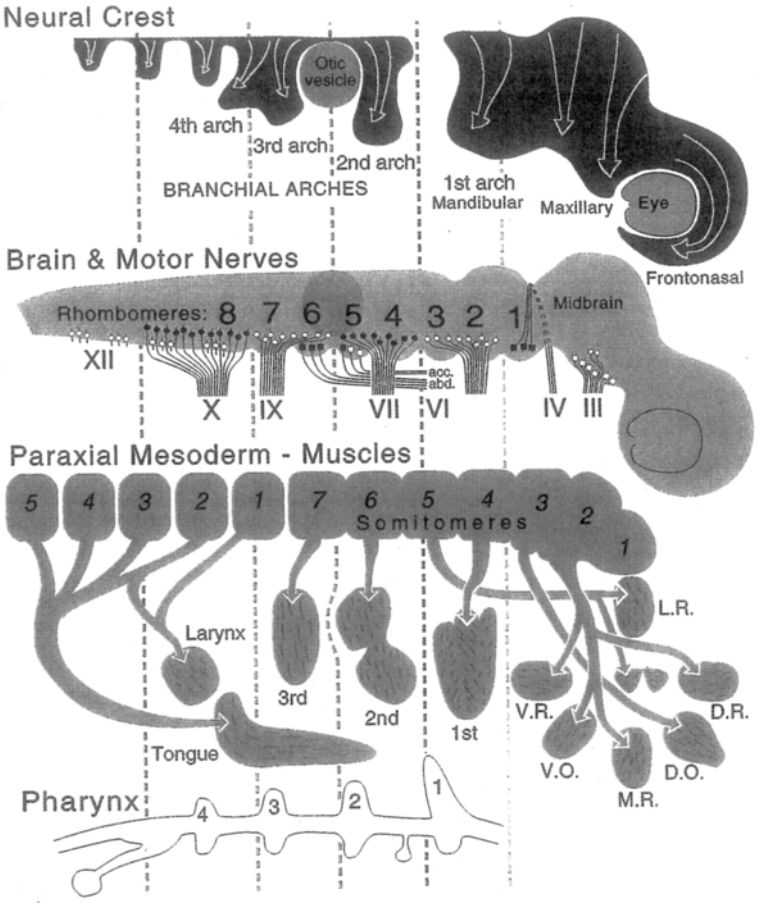

Fig. 9.7 Locations of the craniofacial precursors (Reproduced from Rubin et al. [2])

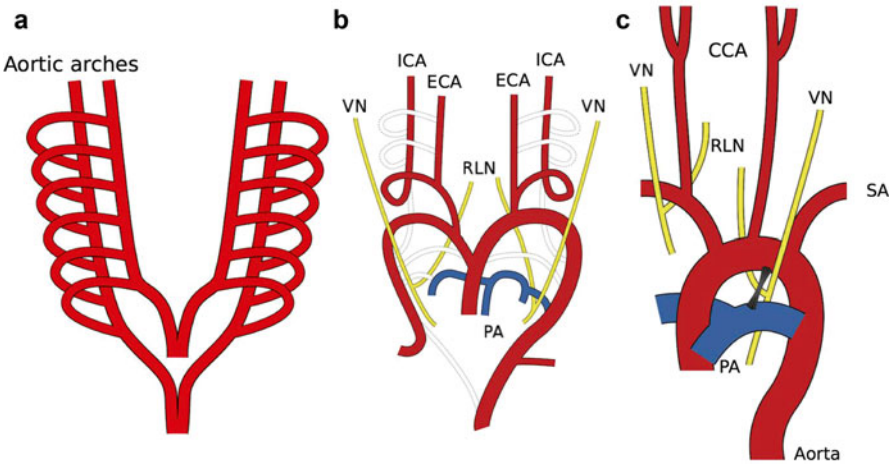

Fig. 9.8 Development of recurrent laryngeal nerve. Aortic arches and dorsal aortas before transformation (**a**), after transformation (**b**), and the recurrent laryngeal nerves and vessels in the adult (**c**). *CCA* common carotid artery, *ICA* internal carotid artery, *ECA* external carotid artery, *SA* subclavian artery, *VN* vagus nerve, *RLN* recurrent laryngeal nerve

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Chapter 10
Laryngeal Framework Regeneration

Masaru Yamashita, Yoshiharu Kitani, and Shin-ichi Kanemaru

Abstract The laryngeal framework consists of complex tissues including the thyroid cartilage, cricoid cartilage, and arytenoid cartilage. This framework contributes to protecting the inner aerodynamic tract from external impact and aids in maximizing vocal fold motion through intra-laryngeal muscular contraction. This structure is affected by malignant tumors, laryngeal trauma, congenital anomalies, stenosis, or intractable inflammatory diseases. Once this rigid structure is damaged, restoration of mechanical power sufficient to compensate for normal laryngeal function is quite difficult. Conventionally, laryngeal defects *have been* reconstructed with autologous tissues or flaps. These reconstructive surgeries, however, required donor tissue, skilled technique and multiple surgeries. Locoregional blood supply also had to be carefully considered to maintain living donor tissue. Tissue regeneration strategies for the laryngeal framework, therefore, have been sought to alleviate these problems. Recently, tissue engineering has attracted great attention as a means of recreating organs. There are three fundamental components in tissue engineering: cells, scaffolds, and growth factors. Among these, scaffolds play a central role in laryngeal framework regeneration because great mechanical power is required immediately after surgery to maintain airway structure. In situ tissue engineering techniques, which allow in vivo regeneration of organs through the application of scaffolds, have shown recent advancement due to biomaterial innovations. In this chapter, current progress and limitations of laryngeal framework regeneration will be discussed. To date, intraluminal epithelialization and subepithelial tissue regeneration have achieved some success after laryngeal resection. Research into the next steps, including functional tissue regeneration and development of suitable scaffolds for children, is now warranted.

Keywords Larynx • Framework • Cartilage • Regeneration • Tissue engineering • Scaffold

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10.1 Background

The laryngeal framework protects the intricate inner structure, the vocal folds, which are finely controlled during phonation, breathing, and swallowing. This framework is composed of a cartilaginous complex including the thyroid cartilage, cricoid cartilage, and arytenoid cartilage. This framework not only contributes to protecting the inner aerodynamic tract from external impact, but also aids in maximizing the effects of the underlying vocal fold motions through intra-laryngeal muscular contraction.

Treatment for various disease conditions such as malignant tumors, laryngeal trauma, congenital anomalies, stenosis, or intractable inflammatory diseases may require resection of the laryngeal tissue. This results in persistent postoperative problems in voice production and/or swallowing. Thus, a conflict exists between effectively treating tumors and functional restoration when handling laryngeal malignancies. Once this rigid structure is damaged, it is very difficult to restore the mechanical power needed to compensate for normal laryngeal function as well as *to* restore the barrier between the inner lumen and subcutaneous tissue. Furthermore, factors including airway pressure changes between inspiratory negative pressure and expiratory positive pressure, saliva from the pharynx, laryngeal secretions, and nonsterile conditions may all contribute to *a* decreasing rate of success during laryngeal framework reconstruction.

Laryngeal defects have conventionally been reconstructed with various autologous tissues or flaps, including muscle flaps [1–3], myocutaneous flaps [4, 5], thyroid gland flaps [6, 7], and cartilage grafts [8–10], among others. These reconstructive surgeries, however, often require donor tissues, highly skilled techniques, and multi-staged surgeries. Locoregional blood supply must also be carefully considered in order to maintain living donor tissue. The scope of these surgeries was generally limited to complete "tissue obliteration" of the extirpated area.

Laryngeal transplantation has also been studied in cases of hemilaryngectomy [11] and total laryngectomy [12–15] in various animal models. In 1998, a successful case of human laryngeal transplantation was reported [16]; however, many barriers remain before laryngeal transplantation becomes a standard treatment. Some of these challenges include managing donor larynges, potential tissue-borne transmitted diseases, ethical issues, and the inevitable administration of immunosuppressive reagents, which could lead to malignant tumor progression.

Regenerative medicine has made great strides in sparing clinicians from the above-mentioned problems, with the goal of repairing affected organs using live cells or tissues without the need for donor tissues. There are two major approaches in regenerative medicine, one is cell therapy and the other is tissue engineering. Beginning in the early 1970s, cell culture for therapeutic purposes was studied using chondrocytes [17]. While cell therapy is appropriate for hematopoietic diseases, it is very difficult to mechanically sustain even moderately sized defects using cell pellets or cell clusters. Tissue engineering has thus emerged to solve this issue and is becoming widespread in the medical field. The basic concept of tissue

engineering was proposed by Langer and Vacanti in 1993 [18]. Their approach was to create tissue ex vivo with the use of three fundamental components: cells, scaffolds, and regulatory growth factors, under the appropriate environmental conditions. The intent was to subsequently implant the created tissue into a live recipient at a later time. When considering laryngeal framework regeneration, scaffolds are considered to play the most important role of *these* three fundamental components, because great mechanical power is necessary immediately after resection surgery in order to maintain airway structure.

In contrast to the original concept of ex vivo tissue engineering, an "in situ" tissue engineering technique has been developed, which allows for the regeneration of organs in vivo by introducing an appropriate scaffold for migrating cells from regions surrounding the affected site. This technique was first proposed and investigated in canine models beginning in 1995 in studies involving the trachea [19, 20], stomach [21, 22], and small intestine [23]. Omori et al. reported the first human case of tracheal regeneration using this technique in 2005 [24]. Polypropylene, a commonly used plastic material, was used in the study. The achievements in this field were mainly attributed to innovations in the available biomaterials. This technique eliminates the time required for harvesting organs that are created ex vivo as well as the need for repeated highly skilled surgeries. With in situ tissue engineering, patients can undergo repeated replacement surgeries if needed in cases where the initial treatment results in failure. Although infection control is a major consideration when using scaffolds, a wide variety of biomaterials, together with improvements in tissue biocompatibility, have greatly aided this problem.

The key to success in regeneration of the laryngeal framework is based on designing ideal artificial scaffolds. Once the ideal scaffolds have been identified, modifications *intended* to enhance regeneration by the addition of cells and/or regulatory growth factors can be considered, assuming strict quality control and patient safety as prerequisites.

10.2 Scaffolds: The Innovation of Bioartificial Materials

In situ tissue engineering studies on the laryngeal framework have mainly been conducted using canine models [25–31]; this is because the structure and size of the canine laryngeal framework is similar to that in humans. The scaffolding materials utilized in these studies to date can be divided into two types: (1) chemically or mechanically decellularized materials used as allogeneic or xenogeneic grafts and (2) synthetic materials. The incorporation of cells into scaffolds is necessary to create living tissues. Appropriate inflammatory responses at the surgical site and the integration of the scaffolds with stem cells or other differentiated cells are presumably enhanced by regulatory growth factors as part of the regenerative cascade. During the normal repair process in injured tissues and organs, endogenous factors are secreted at the damaged sites and serve as regulatory factors [32].

10.2.1 Decellularized Materials

Huber et al. [25] reported the utility of porcine-derived xenogeneic extracellular matrix (ECM) for canine laryngeal framework regeneration in 2003. This scaffold, made from porcine urinary bladder submucosa ECM (UBS-ECM), is decellularized bladder tissue and contains not only extracellular components but growth factors as well, namely, vascular endothelial cell growth factor, basic fibroblast growth factor, and transforming growth factor β [33, 34]. Since this material is acellular and degradable, it was stated to have low antigenicity. Huber et al. made a partial hemilaryngectomy in 30 dogs and applied multiple layers of UBS-ECM for the framework and a single layer of UBS-ECM for the vocal fold prominence. Histological data demonstrated that rapid ingrowth of mononuclear and polymorphonuclear cells from surrounding tissue occurred within 1 week, followed by scaffold degradation and replacement by recipient tissue within 2 months postoperation. Epithelialization was completed by a simple squamous cell layer *by* the first postoperative month. Cartilage, skeletal muscle, and glandular tissues without chronic inflammation were later identified and persisted through the 12-month follow-up period. Although the components were not well-structured and the length of the regenerated vocal fold was shortened compared to the naïve fold, this study clearly demonstrated that porcine-derived ECM scaffolds can be utilized in laryngeal framework regeneration.

Ringel et al. [35] have reported the detailed characteristics of this urinary bladder ECM. The surface of the original ECM was designed to be implanted toward the luminal side to enhance epithelial growth, while the opposite side, the coarsely textured tunica propria surface, was designed to provide enhanced conditions for vascularization and host cell migration. Anatomical and histological examinations were performed in two dogs 24 weeks after hemilaryngectomy; one was reconstructed using ECM and the other with a strap muscle flap. Although the sample size was small, the hemilarynx reconstructed with ECM showed better results than the hemilarynx reconstructed with a strap muscle flap.

Kitamura et al. [31] also used this ECM in a canine hemilaryngectomy model (Fig. 10.1a–c) and evaluated the animals 6 months postoperatively; five dogs were utilized for this study. Functional analyses including vibratory and phonation threshold pressure were performed along with endoscopic and histological examinations. Normal to near-normal phonation threshold pressure and normalized mucosal wave amplitude were observed in approximately half of the dogs. Endoscopic results showed the formation of granulation tissue within 1–2 weeks; one case of infection was reported and resolved within 2 weeks. Re-epithelialization was complete in every dog by 1 month, which was consistent with the previous report [25]. A macroscopic intraluminal view showed a good prominence at the surgically resected site 6 months postoperatively (Fig. 10.1d). Histological data also revealed the partial appearance of newly generated cartilage and muscle tissue within the original portion of the larynx (Fig. 10.1e). The vocal fold was also regenerated; however, fibrotic contraction and the absence of a layered structure ~~was~~*were* noted in the lamina propria, and was defined as a "scarred vocal fold". The data thus

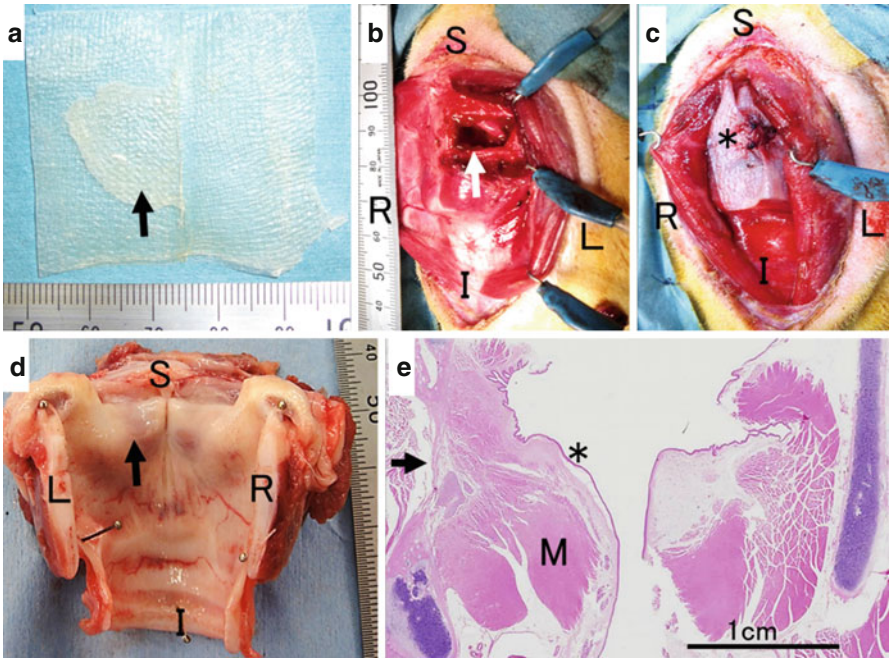

Fig. 10.1 (**a**) A designed porcine urinary bladder-derived ECM. An *arrow* indicates a flap for a vocal fold ridge. (**b**) A left partial hemilaryngectomy was made as indicated by an *arrow. S* superior, *I* inferior, *R* right, *L* left. (**c**) An *asterisk* indicates the implanted scaffold. (**d**) An intraluminal image 6 months after the operation. An *arrow* indicates the surgically affected site (left vocal fold). (**e**) A hematoxylin- and eosin-stained image indicates regenerated epithelium (*asterisk*), muscle tissue (*M*), and cartilage (*arrow*) at 6 months postoperatively

support the use of ECM scaffold as a promising tool for both physical and functional regenerative surgeries after laryngeal resection, although further studies are warranted.

Recently, *a* decellularized laryngeal scaffold has been explored as a new material for laryngeal regeneration [36, 37]. Hou et al. [37] showed the potential for laryngeal regeneration using a chemically decellularized whole rabbit larynx. They found that the scaffold contained few intact cells and, when implanted into the omentum after recellularization with mesenchymal stem cells, vascularization was clearly seen within 4 weeks after implantation and integrated cartilage frameworks remained after 8 weeks.

Decellularized (acellular) materials are useful tools for laryngeal framework regeneration. Decreased antigenicity contributes to reducing the immune-allergic reaction, excessive inflammation and rejection of scaffolds. Biodegradable scaffolds are also ideal when considering the long-term side effects caused by implanted materials. In the future, regenerative surgeries using biodegradable scaffolds may be preferred for use in children and adolescents, once the mechanical strength of these *materials has* been fully established.

10.2.2 Synthetic Materials

Various studies on tracheal prostheses have been conducted since the 1960s; however, the materials used have not been ideal. Nakamura et al. [19, 20] reported successful tracheal regeneration in canine models using polypropylene-based artificial scaffolds, which were designed to be infiltrated with native recipient tissue based on the concept of in situ tissue engineering. This scaffold *was* composed of a cylindrically shaped polypropylene mesh framework with spongy porcine dermal collagen. Polypropylene is a commonly used plastic material. This type of polypropylene mesh has already been utilized widely in the clinical setting as a permanent and nondegradable implant for the surgical repair of abdominal herniation cases. This polypropylene mesh has benefits in terms of its high biocompatibility and morphological flexibility. The polypropylene mesh framework is covered in freeze-dried spongy collagen derived from porcine skin. This collagen is composed of 70–80 % collagen type I and the remaining 20–30 % of collagen type III. This collagen treatment is the key to better cellular attachment and sealing off from the airway after the preclotting procedure with autologous peripheral blood during surgery. This preclotting procedure can enhance the processes of tissue regeneration when bone marrow aspirates or bone marrow–derived stem cells [38] are applied to the scaffolds. Nakamura et al. [38] observed faster epithelialization and fewer complications in these types of regenerative experiments in canine trachea.

Following the successful use in tracheal regeneration, Omori et al. [26] applied this material to cricoid regeneration in a canine model. They utilized a total of nine dogs for cricoid cartilage resections ($n=5$) and cricoid with cervical tracheal resections ($n=4$). Two animals showed minor formations of granulation tissue and one showed exposure of the mesh framework; however, these lesions were asymptomatic. Mechanical tests of the regenerated cricoid showed equivalent mechanical strength *comparable* to native cricoid cartilage. Favorable luminal epithelialization of this material was observed 6 weeks after implantation. Regeneration of ciliated epithelium was confirmed by scanning electron microscopic examination.

Omori et al. [24] then applied this material to four patients in an attempt to repair the cricoid and trachea, including, in one case from 2002, a portion of the thyroid cartilage. One case of subglottic stenosis and three cases of thyroid cancer underwent in situ tissue engineering surgeries using this scaffold. During the 8–34-month observation period, every case showed a well-epithelialized airway lumen without any obstruction or major complication. Their study thus indicated that this technique could be a useful tool for laryngotracheal reconstruction, which *had* sometimes resulted in failure due to granulation, scar, and fibrosis. It took approximately 2 months to achieve sufficient luminal epithelialization in this study. Since the polypropylene-based scaffold is nonbiodegradable and free from growth factors, in situ tissue engineering of the cricoid and trachea may be a good indication for adult cases with stenosis and malignancies. Moreover, the adjustable mechanical power suits the need for sustaining inner airway structure, although this material cannot be utilized in children yet because of its rigid and nonbiodegradable structure.

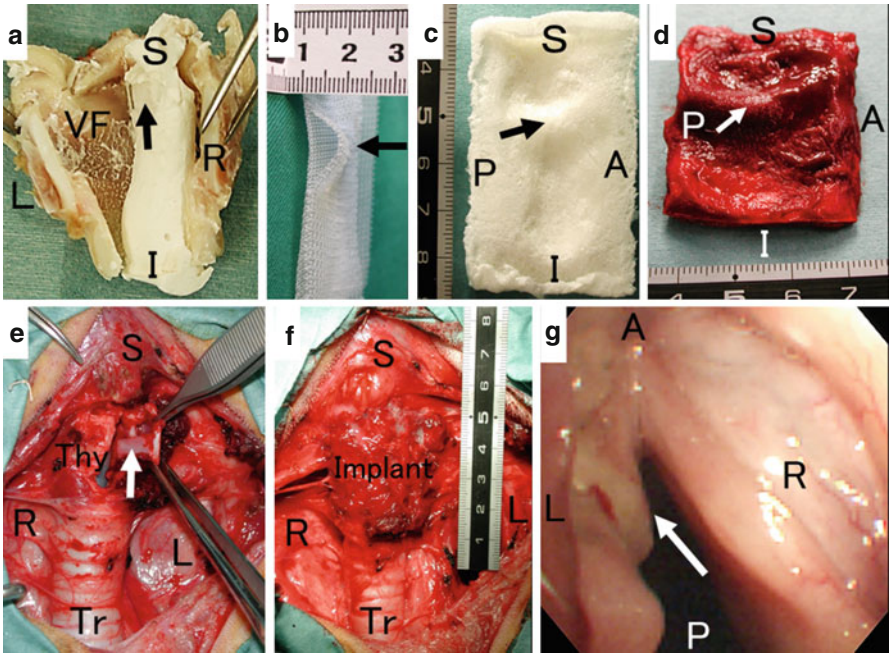

Fig. 10.2 (**a**) Casting procedure. The intraluminal shape of left vocal fold (*VF*) was reflected on the cast (*arrow*). *S* superior, *I* inferior, *R* right, *L* left. (**b**) Contours of the vocal fold were replicated on a dual-layered polypropylene scaffold (*arrow*). (**c**) An image of the scaffold covered with freeze-dried spongy collagen. *P* posterior, *A* anterior. (**d**) An image after preclotting procedure with BSCs and peripheral blood. (**e**) A left hemilaryngectomy was performed. An *arrow* indicates an original left vocal fold. *Thy* thyroid cartilage, *Tr* trachea. (**f**) The scaffold implant was anastomosed to the surgical defect. (**g**) A fiberscopic image taken 3 weeks after the operation. A left vocal protrusion covered with epithelium was observed

Kanemaru et al. [39] reported the combination of this polypropylene-based scaffold *in conjunction* with a growth factor, basic fibroblast growth factor (b-FGF), in three clinical cases of cricotracheal stenosis after long term intratracheal intubation. Although the follow-up period only lasted up to 6 months, they showed preferable airway regeneration using the scaffold combined with *the* growth factor. The slow-release effect of b-FGF in combination with the surrounding spongy collagen was thought to enhance angiogenesis in the scaffold. Given the reduced possibility of tumor formation after local b-FGF application during the short postoperative period, this tissue engineering technique may be a viable approach for cases with cricotracheal stenosis.

Yamashita et al. [27] revised the polypropylene scaffold framework for the subsequent regeneration of the thyroid cartilage. Replication of the intricate luminal shape of the canine larynx was performed using a dental cast (Fig. 10.2a). Two-ply polypropylene mesh sheets with similar concavity and convexity of the vocal folds were designed (Fig. 10.2b). In preliminary studies, the sheets were coated with spongy collagen (Fig. 10.2c) and were implanted after hemilaryngectomy in their

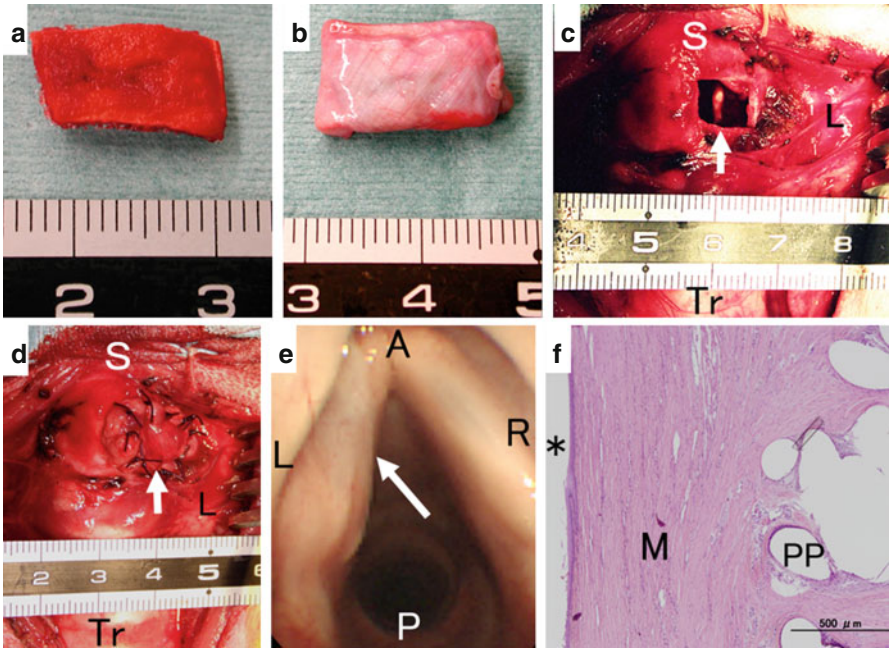

Fig. 10.3 (**a**) A polypropylene-based scaffold preclotted with peripheral blood. (**b**) The scaffold was covered with autologous fascia lata. (**c**) Membranous portion of left vocal fold was resected through a window defect created in a left ala of thyroid cartilage. *S* superior, *Tr* trachea, *L* left. (**d**) The scaffold implant was anastomosed to the resected site. (**e**) A fiberscopic image taken 3 months after the surgery. A preferable luminal shape with epithelialization was obtained at the surgical site. *R* right, *A* anterior, *P* posterior. (**f**) A hematoxylin- and eosin-stained image 3 months postoperatively. Luminal surface is covered with squamous epithelium (*asterisk*). Sparse muscle tissue is observed (*M*) between epithelium and scaffold. No inflammatory reaction is seen. *PP* polypropylene framework

canine model; these initial attempts resulted in failure, even though the same methods were successfully used for cricoid and tracheal regeneration. This failure might be due to movements resulting from swallowing and barking as well as infection of the scaffold from secretions which are inevitable because of the anatomical location. Next, they utilized bone marrow-derived stromal cells (BSCs) as an enhancer of the regenerative process and peripheral blood for preclotting the scaffold (Fig. 10.2d) for their hemilaryngectomy model (Fig. 10.2e, f). One out of three dogs showed favorable epithelialization with preferable luminal contour (Fig. 10.2g), although no histological assessment was performed. While a beneficial contribution of BSCs was observed, it is still unknown how they behaved in the scaffold in vivo.

Yamashita et al. [29] reported in situ tissue engineering of the canine thyroid cartilage after a partial window defect (size of 1.2×0.7 cm). In the experimental group ($n = 5$), they utilized a single polypropylene mesh sheet with spongy collagen covered with autologous fascia lata after preclotting with peripheral blood (Fig. 10.3a–c), and in the control group ($n = 3$) a strap muscle flap was used for their

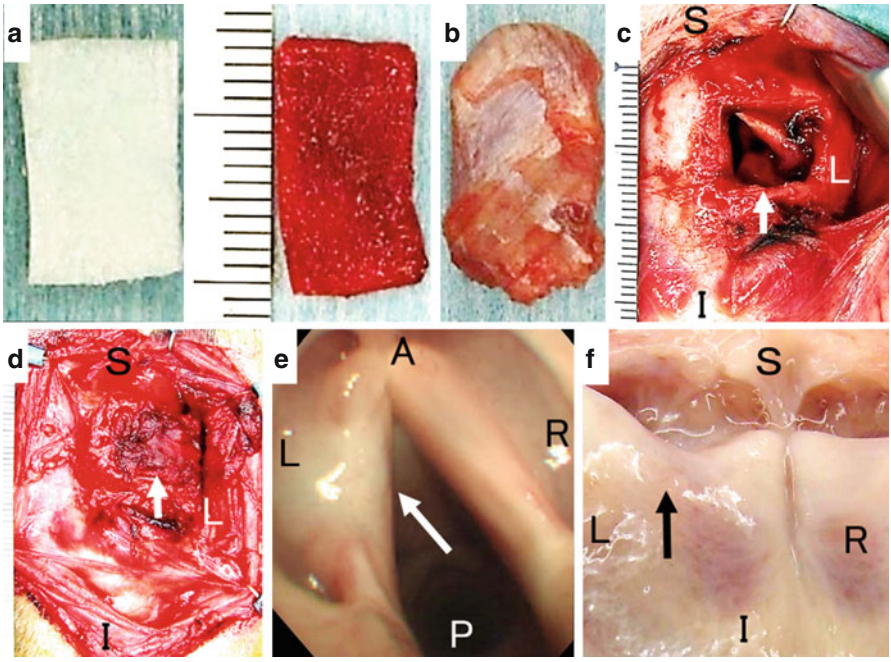

Fig. 10.4 (**a**) A polypropylene-based scaffold covered with spongy collagen. (**b**) The scaffold after preclotting procedure. (**c**) Autologous fascia lata was used to cover the preclotted scaffold. (**d**) A left partial hemilaryngectomy was performed (*arrow*). *S* superior, *I* inferior, *L* left. (**e**) The scaffold implant was anastomosed to the defect site. An *arrow* indicates the scaffold implant. (**f**) A fiberscopic image taken 6 months after the operation in 3 % collagen group. An *arrow* indicates regenerated left vocal prominence. *A* anterior, *P* posterior, *R* right. (**g**) A macroscopic intraluminal image from excised larynx revealed difference in vertical levels of vocal folds (*arrow*), which resulted in suboptimal vibratory data

window defect model (Fig. 10.3c, d). Four out of five dogs showed good vocal fold eminence under fiber-optic examination; typical cases showed residual tissue of the fascia covering until the 7th postoperative day and epithelialization was completed within 1 month (Fig. 10.3e). The histological data at 3 months postoperation revealed the regeneration of lined epithelium, subepithelial tissue, and muscle (Fig. 10.3f) in both groups without any new cartilage formation. Vibratory assessments from the experimental group showed suboptimal results. The protective effects from infection at early time points seemed very important for obtaining favorable regeneration.

Kitani et al. [30] reported on a canine partial hemilaryngectomy model (with a size of 1.8×1.0 cm) and compared the 1 % classical type ($n=6$) and 3 % new type ($n=6$) of spongy collagen which covers the polypropylene mesh framework (Fig. 10.4a, b). Fascia lata was also used for their artificial scaffolds (Fig. 10.4c). While the 1 % collagen model showed a half successful ratio without mesh implantation, the 3 % collagen model showed successful results in all cases after

hemilaryngectomy (Fig. 10.4d–g). They stated that the 3 % collagen model contributed to tissue regeneration with harder mechanical stiffness and slower absorption than the 1 % collagen model. Histological data indicated the presence of a fine epithelial lining with subepithelial tissue without any cartilage formation in successful cases. Vibratory data were suboptimal mainly due to a difference in vertical levels of the regenerated vocal folds.

Metallic substances have also been investigated for cricoid regeneration. Tan et al. [40] examined the efficacy of a porous metastable β-type titanium alloy for regeneration of the cricoid and trachea (20 mm in length) in 10 mongrel dogs. The titanium alloy with tiny pores (diameters of about 70–90 μm) was designed to a final porosity of 30–35 %. The final prostheses showed a cylindrical C-shape and were 0.5 mm thick, 20–22 mm in inner diameter, and 20 mm in length. Two dogs died of an accidental complication with anesthesia and pneumonia. Granulation was observed in four cases out of eight, and one showed exposure of the metastable β-type titanium alloy plate, although these animals were asymptomatic. During the 3–8-month postoperative follow-up period, all of the prostheses had completely incorporated into the host tissue and histological data showed favorable epithelial lining with simple squamous cells at the midposition and ciliated columnar cells near the anastomoses of the prostheses. The mechanical power of this material was sufficient to prevent airway collapse; however, the scaffolds had to be designed and prepared prior to the regenerative surgeries. This means that the resection area must be precisely estimated before surgery. This study showed remarkable progress in manufacturing metallic scaffolds and their potential application as bioartificial materials. As they stated in the article, further studies related to the long-term outcomes and regenerative surgeries for larger defects should be performed in the future.

What is the ideal scaffold for laryngeal framework surgery? No one yet knows the answer. Extensive study into the various scaffolds should be performed. Major factors to take into consideration include materials, processing techniques to improve cellular attachment and/or ingrowth on the surface of the scaffolds, lower immune-rejection and antigenicity, better biocompatibility, and easier handling with sufficient mechanical power. Bioabsorbable scaffolds, which will be substituted with host cells over the long term, would be preferable if they could retain sufficient mechanical properties to prevent collapse of airway structures. These scaffolds could thus be applicable to stenotic lesions in children.

10.3 Cells and Regulatory Growth Factors

In 2000, Wambach et al. [41] reported potential cartilage regeneration in vitro using chondrocytes from canine thyroid cartilage and bovine-derived type I collagen matrix. The isolated and cultured chondrocytes grown on bovine type I collagen expressed type II collagen, which was produced by the chondrocytes. No gross cartilage formation was noted in this study. As of now, in order to generate

mechanically durable gross tissue using chondrocytes, other scaffold templates for chondrocytes or an in vivo prefabrication period is likely required.

Katic et al. [42] examined the regenerative effects on thyroid cartilage defects (1.5 cm^2) without opening the lumen in a canine model using thyroid cartilage allografts and human recombinant osteogenic protein-1 (OP-1; bone morphogenetic protein-7). The data revealed that OP-1 combined with thyroid cartilage allografts induced bone, cartilage, and ligament-like structures comprising up to 80 %. The borders of the defects were shown to have healed by formation of new bone in cases where bone resided within the old thyroid cartilage layers.

Tcacencu et al. [43] published an article describing the regeneration of partial defects (2 mm) in the anterior portion of the rabbit cricoid cartilage utilizing recombinant human bone morphogenetic protein-2 (BMP-2) and collagen sponge as a carrier. They found new cartilage and bone formation 4 weeks after surgery when using BMP-2 with collagen sponge. No discontinuity at the boundaries of the implant was observed. Proteoglycans were also produced by the new cartilage.

Thus, regulatory growth factors are clearly able to modulate the tissue regeneration process, but may have unintended effects on cell behavior.

Nomoto et al. [44] reported on the effects of allogeneic dermal fibroblasts on polypropylene mesh with collagen sponge for cricoid regeneration in rats. In the group treated with heterotopic fibroblasts, epithelialization and changes in collagen fibers occurred more rapidly than in the scaffold group without cells. Although the fate of allogeneic dermal fibroblasts and the mechanism by which they accelerate tissue regeneration are unclear, this study indicated that the addition of cells could also directly modulate the tissue regeneration process.

When ex vivo cell culture is employed in clinical use, strict quality control is essential. Contamination by unintended cells or microorganisms and the risks of transmitted diseases from donors or culture serum may hinder its rapid progress in the field of regenerative medicine.

10.4 Future Perspectives

The recent advances in regeneration of the laryngeal framework are remarkable and very promising.

Clinical use of in situ tissue engineering has been applied in cricoid or cricotracheal regeneration since 2002 [45, 39]. Favorable epithelialization and tissue regeneration was achieved in canine studies with maximum resection size *during* hemilaryngectomy at the level of thyroid cartilage [30, 31].

Approaches utilizing tissue regeneration may be more preferable than the complicated and skilled repeated surgeries that involve damage to donor sites in order to reconstruct deficits in the laryngeal framework. To establish these approaches as standard treatment options, current techniques should be modified and refined.

Researchers should focus on the technological innovations pertaining to new scaffolds. Ideal scaffolds may eliminate the need for exogenous cells or growth

factors, making it easier to be applied in clinical practice. Rapid sealing of the defect site from the airway and subsequent epithelialization could also reduce the risk of infections, which is key for obtaining favorable results.

The use of growth factors could be accepted in limited situations. Thoughtful consideration should be taken to prevent tumorigenesis or relapse of malignant tumors. Release time, concentration, and duration could be controlled with the use of carrier compounds. If scaffolds could be successfully excluded from the immunorecognition system, immunomodulation with cytokines or other molecules combined with scaffolds could be beneficial after regenerative surgeries.

When considering the addition of exogenous cells to scaffolds, it becomes much more complicated to predict the types of interactions which could occur within host tissues and scaffolds. Progenitor or stem cells might differentiate into other cell types and might produce different factors over time. Embryonic stem cells and induced pluripotent stem cells might induce tumorigenesis, as they have the potential to become many different cell types. Bone marrow–derived stem cells and adipose-derived stem cells are *also* candidates for regeneration of mesenchymal tissues. Differentiated cells, including chondrocytes or fibroblasts, should not persist as long as stem cells; thus, they are generally considered to be safe due to their rapid disappearance. In general, cell culture media that includes serum enhances cellular activities, i.e., proliferation and differentiation; however, these sera are generally autologous or derived from other animals. Serum-free medium is one option to prevent donor-borne transmitted diseases; however, the proliferative ability of the cultured cells would be greatly reduced.

Once the long-term safety of these techniques is established, the cloning of other mammals for donor tissue harvest might surface for debate, although lifelong immunosuppressive treatments and ethical issues related to the lives of donor animals are inevitable.

The eventual goal of laryngeal framework regeneration is the fully functional restoration of the vocal folds. In order to achieve this goal, many challenging tasks lay ahead, namely, laryngeal re-innervation of both sensory and motor nerves, regeneration of the viscoelastic properties of the lamina propria, and vibratory function through muscle contraction, among others. Further ongoing studies will be required in order to restore laryngeal function in patients with intractable diseases.

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Chapter 11
Vocal Fold Development

Tomoko Tateya and Ichiro Tateya

Abstract The specialized microanatomy of the vocal folds is essential for the voice production. Intimate knowledge of vocal fold histology is fundamental for understanding voice disorders and necessary for approaching treatment. This chapter is focused on how the histologic features of human vocal folds are developed from the fetal period. The process of vocal fold development is mainly characterized by two events: the organization of fibrous proteins that leads to the formation of vocal ligament, and the appearance of macular flavae. Recent contributions to our growing understanding of these events are addressed. The factors that promote vocal fold development are also discussed and lastly a future direction of vocal fold histophysiology is suggested.

Keywords Macular flavae • Vocal fold • Development

11.1 Introduction

Embryogenesis of the human larynx occurs between the 4th and 10th week of fetal life, as discussed in Chap. 9. Vocal fold development occurs late in comparison to other structures in the larynx, such as cartilage and muscles [1]. At about 6 weeks of embryonic age, the primitive laryngopharynx has become compressed bilaterally along its ventral aspect, called the epithelial lamina. The primitive glottis is temporary obliterated. The glottis appears in fetal period, after recanalization of the lumen at about the age of 10 weeks. There remains a pair of lateral recesses (laryngeal ventricles) and both true and false vocal cords differentiate around them [2].

Because of the paucity of specimens and poor postmortem quality of larynges, studies on ex vivo fetal, infantile, and pediatric vocal folds are limited. Consequently,

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much more is understood about vocal fold histology and composition in adults than in neonates or children [3]. However, several distinctive features of fetal, infantile, and pediatric vocal folds different from adults have been described, suggesting that vocal fold development and maturation continues from the time of initial appearance in fetal period to adulthood.

11.2 Development of Fibrous Proteins in Pediatric Vocal Folds

As discussed in Chap. 12, the cover-body theory of phonation explains how the layered structure of the lamina propria contributes to the human voice [4–6]. The theory proposes that the five layers of adult vocal folds can be reclassified into the cover (which consists of the epithelium and the SLLP), the transition zone (which includes the ILLP and the DLLP), and the body (the vocalis muscle). Subsequent studies have endeavored to clarify the structural organization of the extracellular matrix of the vocal fold lamina propria that enables the vocal fold to vibrate with consistency and control. Now it is widely accepted that the distribution of fibrous proteins such as collagen and elastin in the extracellular matrix characterizes three layers of vocal fold lamina propria, and the organization of fibrous proteins in these layers is directly related to the viscoelastic and biomechanical properties important for vocal fold vibration [7].

The three-layered structure of lamina propria is a feature specific to adult human vocal folds, and the fetal and infant vocal fold has been considered to lack this structure on the basis of existing histologic evidence. Hirano [8] and Kurita [9] reported that the lamina propria had a uniform appearance under the light microscope in the vocal folds of fetuses and neonates. Sato et al. also reported that in newborn vocal folds observed by light and electron microscopy the entire lamina propria appeared as a uniform structure with no vocal ligament or no layered structure, and Reinke's space of the newborn vocal fold was a loose structure composed of ground substance and sparse fibers [10, 11]. They described fibrous protein organization as postnatally occurring and increasing during childhood, shown in Fig. 11.1 [12]. They stated that after birth the collagenous and reticular fibers extended toward the middle of the membranous portion of the vocal fold mucosa from the maculae flavae and elastic fibers increase after collagenous and reticular fibers appear in Reinke's space [10–14].

Ishii et al. visualized collagen fibers and elastic fibers separately in pediatric cadaveric vocal folds by scanning electron microscopy after digestion treatment with sodium hydroxide and formic acid and noted the development of fibrous protein organization in vocal fold lamina propria by 17 years of age [15] (Fig. 11.2). They reported that the vocal fold lamina propria in fetuses and neonates consisted of sparse and relatively dense areas of collagen and elastic fibers that ran at random and no longitudinal arrangement was found, and the vocal ligament was not found. In subjects 5 years of age, a deep dense area was found in the anterior and posterior maculae flavae, and longitudinal fibers were noted between the maculae. A structure

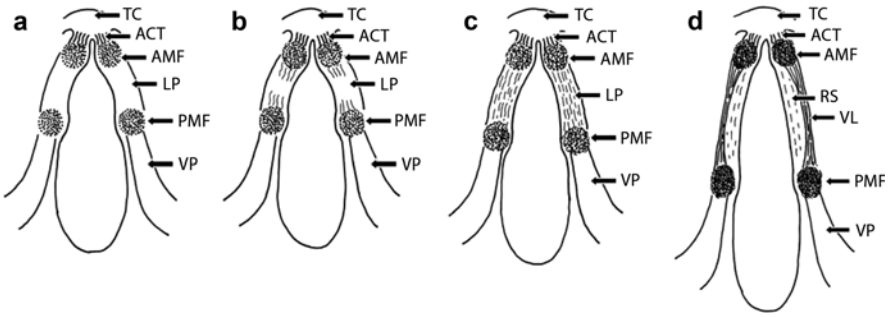

Fig. 11.1 Human newborn (**a**), infant (**b**), child (**c**), and adult (**d**) macula flavae and growth and development of the human vocal fold mucosa. *TC* thyroid cartilage, *ACT* anterior commissure tendon, *AMF* anterior macula flava, *PMF* posterior macula flava, *P* vocal process of arytenoid cartilage, *RS* Reinke's space, *VL* vocal ligament, *LP* lamina propria of the vocal fold mucosa (Reproduced from Sato et al. [12])

of superficial versus deep layers appeared in children older than 10 years of age. The layered structure of the lamina propria was complete around 17 years of age [15] (Fig. 11.2).

Nita et al. studied the fine structure of the vocal folds from human fetuses and neonates and analyzed collagenous and elastic fibers in the lamina propria by using light and electron microscopy [16]. Collagen fibers were viewed using the Picrosirius polarization method and elastic system fibers were stained using Weigert's resorcin–fuchsin after oxidation with oxone. They reported that the distribution of collagen and elastic fibers in the lamina propria of fetal vocal fold resembled that previously described for the adult vocal ligament, suggesting that a vocal ligament has already begun to develop by the time of birth [16].

Thus, there have been discrepancies regarding the composition and orientation of collagen and elastin fibers in pediatric vocal fold lamina propria. It is probably because the methods for visualizing the fibrous proteins used in these reports differed in sensitivity and specification. There seems to be no consensus as to when the vocal ligament first emerges so far. Accumulating evidences indicate that extracellular matrix components such as collagen and elastin are present in vocal fold lamina propria from the fetal period, leaving the question whether such fibrous proteins form the structure that can be called as vocal ligament. It is widely accepted that the organization of fibrous proteins undergoes considerable development after birth until it attains the highly complex structure seen in adults.

11.3 Development of Macula Flavae in Pediatric Vocal Folds

The human vocal fold is known to have macula flavae in both the anterior and posterior ends of its membranous portions and the vocal ligament runs between the anterior and posterior macula flavae, as described in Chap. 13.

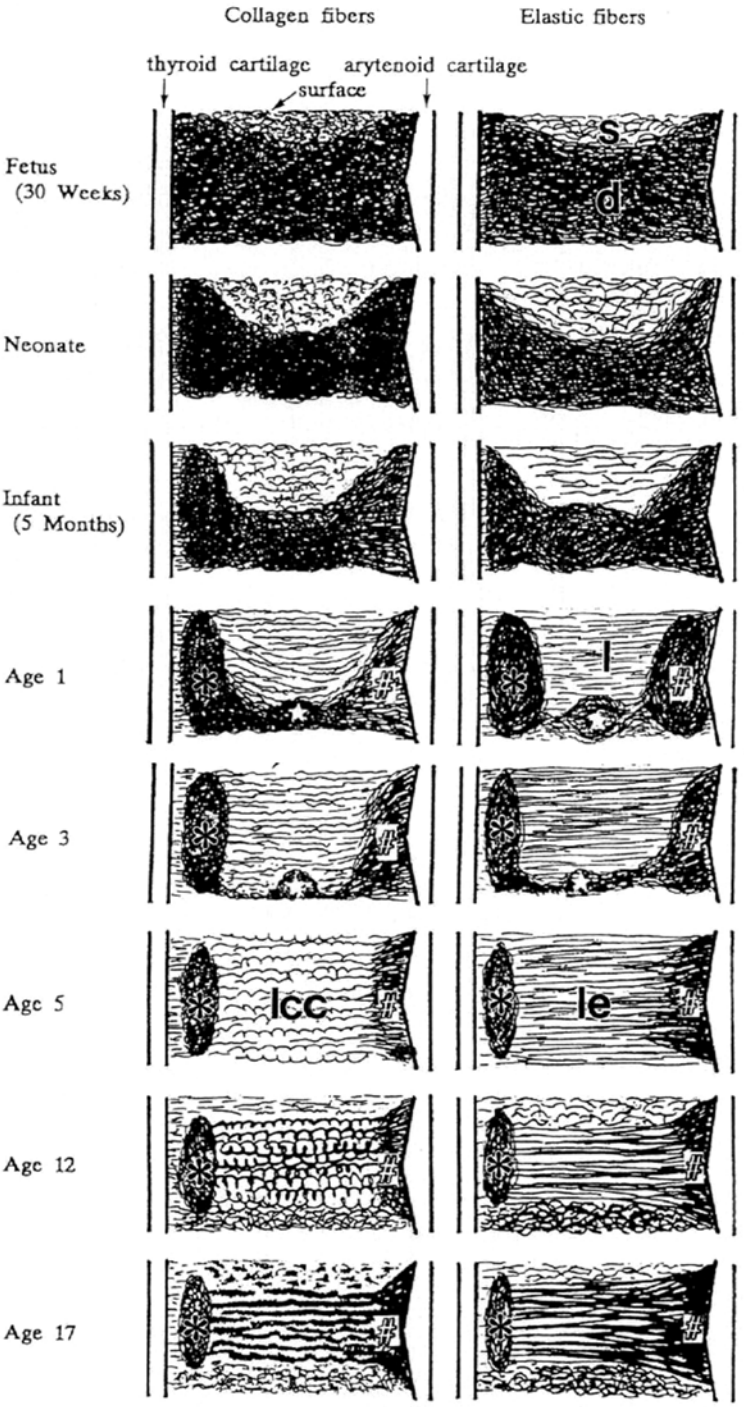

Fig. 11.2 Developmental changes in lamina propria of human vocal fold. *Asterisk* anterior macula flava, # posterior macula flava, *star* vestige of deep part, *d* dense area, *s* sparse area, *l* longitudinal fibers, *lcc* longitudinal curly collagen fibers, *le* linear elastic fibers (Reproduced from Ishii et al. [15])

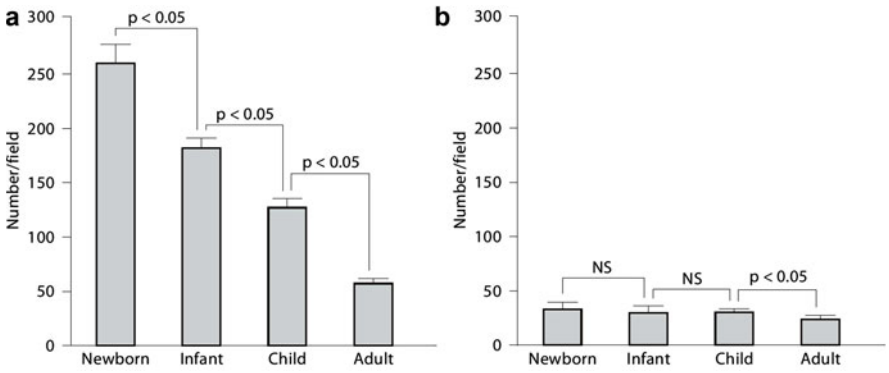

Fig. 11.3 Cell density in the human vocal fold mucosa. *NS* Not significant. (**a**) Vocal fold stellate cell density in the human macula flavae. (**b**) Fibroblast density in the lamina propria (Reproduced from Sato et al. [12])

In the fetal period, macula flavae in human vocal folds develop asynchronically with the posterior macula flavae appearing before the anterior ones [17]. The earliest appearance of posterior macular flavae have been observed at 11 weeks of amenorrhea [17], 13 weeks of amenorrhea [18] or 13.5 weeks of amenorrhea [19], whereas the anterior macula flavae have been observed at 16 weeks of amenorrhea [18, 19] or 18 weeks of amenorrhea [17].

Sato et al. have precisely investigated the morphology of the macula flavae in the human vocal folds [10, 12–14, 20–27]. Based on their studies, adult human macula flavae were composed of dense masses of fibroblasts, elastic fibers, collagenous fibers, and ground substance [12, 20–26]. Fibroblasts in the macula flavae tended to be stellate in shape with smaller nucleus–cytoplasm ratio and stored vitamin A-containing lipid droplets whereas fibroblasts in Reinke's space of intermacular vocal fold mucosa tend to be oval-shaped without lipid droplets [11, 20]. Because of these morphologic differences from the fibroblasts in the human vocal fold intermacular mucosa, Sato et al. designated them as vocal fold stellate cells [23, 24]. They presumed these stellate cells produced extracellular matrix components in vocal fold mucosa.

Sato et al. examined and characterized pediatric vocal fold macula flavae at various ages from neonate [10, 12–14]. According to their reports [10–13], neonatal macula flavae locate at the anterior and posterior ends as well, but the macula flavae appear to be closer to the cartilage. Similar to adult macula flavae, neonatal macula flavae are composed of dense masses of fibroblasts, elastic fibers, collagenous fibers, and ground substance. Vocal fold stellate cells in the newborn macula flavae are stellate or oval in shape and smaller in size, and the nucleus–cytoplasm ratio is higher compared with those of the adult. A few lipid droplets are present in the cytoplasm but they are smaller and fewer than those found in adults. The density of cells in the newborn macula flavae is much greater and about five times that in the adult macula flavae (Fig. 11.3a). These characteristics of neonatal macula flavae have changed with age and become more similar to the characteristics of adult macula flavae [11, 12, 14]. The density of cells in the macula flavae decreases with age (Fig. 11.3a). On the other hand, fibroblasts are relatively sparse in the lamina propria

of the newborn, infant, and child focal folds, though cell density of child lamina propria is significantly higher than that of adult (Fig. 11.3b).

The appearance of collagenous and elastic fibers composing vocal ligament, in and between the macula flavae of adult human vocal folds, suggests the implication that the macula flavae control the synthesis of these fibers [19]. Mainly based on morphological investigations into the macula flavae, the role of vocal fold stellate cells in collagen and elastic fiber synthesis have hypothesized by several authors [17, 21, 22]. However, it is difficult to prove directly that vocal fold stellate cells synthesize fibrous proteins composing the vocal ligament, because the vocal ligament is unique to human vocal folds and the verification of the hypothesis is difficult. So far, there have been only histological observation and there have been no evidences based on prospective, controlled, or experimental study. The functions of macula flavae and stellate cells in human vocal fold development and maintenance are still to be elucidated.

11.4 What Stimulate Vocal Fold Development?

Among mammals, only humans can speak and only human adult vocal folds have layered structure such as vocal ligament and Reinke's space [28]. What are the factors of initiating the development from pediatric vocal folds into adult vocal folds exhibiting specialized microanatomy is still in question.

Tension has been thought to be the most important factor that influences the synthesis of collagenous fibers by fibroblasts [29]. The bending stresses on the vocal folds associated with phonation are greatest in the region of the macula flavae [30]. Sato et al. examined human vocal folds that remained unphonated after birth [31–33]. They reported that the vocal folds do not have a vocal ligament, Reinke's space, or a layered structure, and the stellate cells in macula flavae appeared to have decreased activity to produce extracellular matrix. Therefore, they suggested that the tensions caused by phonation after birth may stimulate the vocal fold stellate cells in the anterior and posterior macula flavae to accelerate production of extracellular matrix and form the vocal ligament, Reinke's space, and layered structure [31–33].

However, several studies have shown that collagen and elastin fibers are observed in lamina propria of fetal vocal folds [15, 17, 19, 20]. Besides the influence of phonation, the genetic control of the fibrous protein syntheses is thought to be of considerable importance.

Vocal folds are known to be androgen and estrogen sensitive. These gonadal steroids influence juvenile vocal fold maturation and result in voice changes from childhood to adulthood that vary in females and males, particularly during puberty [34–36]. Immunohistochemistry studies demonstrate that female and male vocal folds express both estrogen and progesterone receptors [34–37]. Probably because of the gonadal hormones, men and women differ in the pattern of fibrous protein distribution in the vocal folds. Chan et al. showed that significantly higher levels of

collagen were found in the male vocal fold than female both in the cover and the vocal ligament, and in the male vocal folds there was a significantly higher level of elastin in the cover than in the ligament [38]. These differences in fibrous protein distribution seemed to affect the differences in vocal fold property. A higher elastic modulus and stiffer ligament were observed in male focal folds than in female vocal folds [38]. The distribution, pattern, and density of gonadal hormone receptors and their effects on extracellular matrix in the vocal folds remain an area of active research.

11.5 Future Direction

Considering the limitation of human autopsy specimen, not only human studies but also animal studies are fundamental to further understanding of vocal fold development. Toya et al. conducted an interspecies comparison in human, pig, dog, rabbit, and rat vocal fold mucosa [39]. They found that macula flavae and vitamin A-storing stellate cells were not conserved across the vocal folds of all mammalian species but existed in humans and rats [39]. Tateya et al. suggested that rat vocal folds displayed the histological characteristics that were similar, although not identical, to human vocal folds, such as the distribution pattern of extracellular matrix in the lamina propria and clear developmental changes in the morphology of stellate cells and collagen fiber organization [40, 41]. Therefore, rats appear to offer the most appropriate in vivo experimental system of the nonhuman species for modeling of vocal fold biology.

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Chapter 12
Regeneration of the Vocal Fold

Shigeru Hirano

Abstract The vocal fold is the essential mucosa that produces voice. The vocal fold vibrates at a rate of 100–200 Hz up to several hundred Hz. This function is supported by a unique histological layered structure. When this structure is destroyed in cases such as vocal fold atrophy, scarring, and sulcus vocalis, vibratory function is lost and the voice becomes hoarse. The treatment of such voice disorders remains a challenge and a regenerative approach is needed to alleviate this problem. There have been many attempts to regenerate the vocal fold mucosa using tissue engineering strategies including the addition of scaffolding, cell therapy, and growth factor therapy. Regenerative scaffolds include collagen-gelatin-based matrix, hyaluronic-acid-based matrix, and acellular extracellular matrix. Stem cells have received wide attention as a promising tool for inducing tissue regeneration, and mesenchymal stem cells or adipose-derived stem cells have been shown to be suitable for vocal fold regeneration. Growth factors, such as basic fibroblast growth factor and hepatocyte growth factor, have also been demonstrated to have regenerative effects on the vocal fold. Thus, the combination of these components represents a good option for an effective regenerative therapy. This chapter describes the research and clinical application of regenerative treatment of the vocal fold.

Keywords Vocal fold • Regeneration • Tissue engineering • Stem cell • Growth factor • Scaffold

12.1 Normal Structure of the Vocal Fold

The vocal fold is a functional mucosa that vibrates at a high frequency cycle of hundreds of Hertz. This is the only mucosa that vibrates in this manner, resulting in the production of voice. This unique function is supported by a complex histological structure not found in any other mucosa. It is important to be familiar with

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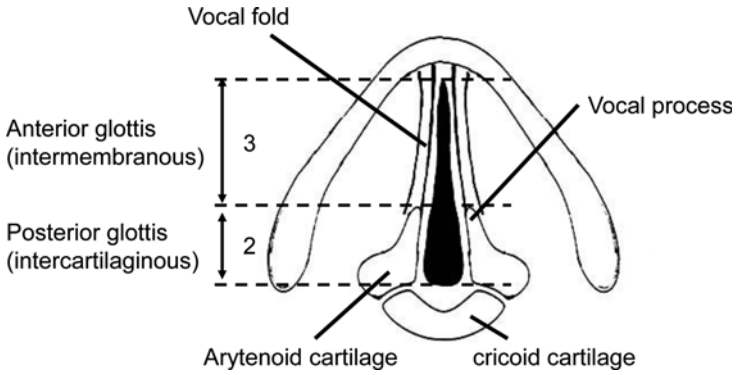

Fig. 12.1 The glottis: anterior and posterior glottis

the microanatomy of the vocal fold when treating patients with voice therapy and/or phonosurgery in order to achieve the most satisfactory outcomes. Once this architecture is destroyed, intractable dysphonia occurs and it is quite difficult to restore the normal structure of the altered vocal fold. Thus, regenerative medicine represents the key to restoring the architecture and function of the vocal fold mucosa.

This chapter will first describe the microanatomy of the vocal fold, the superstructure of the mucosa, distribution of extracellular matrix, and cellular distribution and morphology. Based on this anatomy, a regenerative approach to treatment of the destroyed mucosa, in such cases as vocal fold scarring, sulcus vocalis, or atrophy, is reviewed.

12.1.1 Anatomy of the Glottis

The glottis is divided into two areas by a line between the tips of the bilateral vocal processes: the anterior glottis and the posterior glottis (Fig. 12.1). The anterior glottis includes the vocal folds, while the posterior glottis includes the cartilaginous structures. These two regions are quite different in terms of both structure and function. The anterior glottis, known as the intermembranous portion, is where vocal fold vibration occurs and voice is produced. The posterior glottis, called the intercartilaginous portion, mainly plays a role in respiration. The ratio of the length of the anterior glottis to that of the posterior glottis is approximately 1:1 in newborns, and becomes 3:2 in adults. This has been interpreted to represent the change from newborns that need more respiratory function than voicing, to the shifting need for voice function that is developed with age.

Clinically, more attention is usually paid to the anterior glottis because most lesions that cause dysphonia occur in this region. Therefore, regenerative treatment has chiefly been considered for the anterior glottis, the vocal fold.

12.1.2 Architecture of the Vocal Fold Mucosa

The vocal fold lies between the anterior commissure and the vocal process of the arytenoid cartilages. The vocal fold has unique vibratory properties that are not seen in any other part of the body. These unique properties are supported by its histological architecture, and disruption of the normal architecture causes severe dysphonia that is difficult to treat.

The well-defined layer structure of the vocal fold has been well characterized and consists of the epithelium, lamina propria, and muscle [1, 2] (Fig. 12.2). The epithelium covering the free edge of the vocal fold is a squamous epithelium, while ciliated columnar epithelium covers the supra- and subglottic mucosa. The lamina propria is divided into three layers: the superficial, intermediate, and deep layers. The superficial layer is composed of sparse fibrous tissue and abundant amorphous substances, which makes it more pliable than the other layers. This layer is called "Reinke's space," and it is the main site where mucosal vibration occurs during phonation. Most benign and malignant pathologies affect this region resulting in voice problems. The intermediate layer chiefly consists of elastic fibers, while the deep layer primarily consists of collagenous fibers. These two layers form the vocal ligament, which is connected to the conus elasticus inferiorly and to the anterior commissure tendon anteriorly. The vocalis muscle lies beneath the vocal ligament and forms the main body of the vocal fold. The boundary between the deep layer of the lamina propria and the muscle is not distinct because some collagenous fibers from the deep layer insert into the muscle tissue.

The muscle is the contractile element that supplies a firm scaffold, while the vocal ligament provides a substrate of appropriate stiffness and elasticity on which

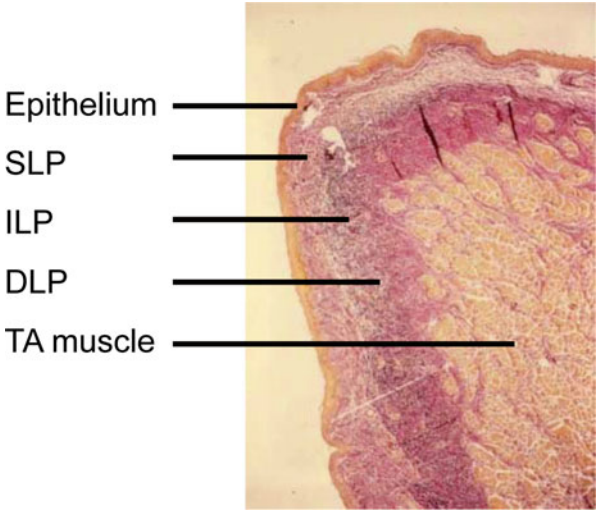

Fig. 12.2 Layer structure of the vocal fold mucosa. The lamina propria consists of three layers: superficial, intermediate, and deep layers. *SLP* superficial layer of lamina propria, *ILP* intermediate layer of lamina propria, *DLP* deep layer of lamina propria, *TA* thyroarytenoide (By courtesy of Minoru Hirano)

the pliable superficial layer vibrates. Stiffness decreases from the muscle to the superficial layer of the lamina propria, that is, the pliability of the tissues gradually increases in the same order. This gradation of the different tissue properties is thought to be ideal for the vibratory function of the vocal fold. This concept is called the "cover body theory," according to which the cover consists of the epithelium and the superficial layer of the lamina propria, while the body consists of the muscle; the intermediate to deep layers of the lamina propria are referred to as the transition [1, 3]. The average thickness of the lamina propria in adults is only 1 mm.

12.1.3 Basement Membrane Zone (BMZ)

The basement membrane zone forms the boundary between the epithelium and the superficial layer of the lamina propria (SLP) and plays an important role in securing this boundary. This zone is directly affected by phonotrauma and any other injuries to the vocal fold. Gray and colleagues have extensively investigated the architecture of the BMZ and its alteration by benign pathology [4, 5]. The basement membrane consists mainly of laminin and type IV collagen. Gray et al. found that type VII collagen fibers connect the basement membrane with the SLP, firmly maintaining the relationship between them; these are called "anchoring fibers." Sato et al. also found type III collagen fibers, called "reticular fibers," in the SLP [6]. The reticular fibers are thin fibrils that may contribute to maintaining the architecture of the SLP. The collagen network consisting of collagen type IV, VII, and III in the BMZ and SLP is thought to be important for maintaining the structure of the "cover."

12.1.4 Extracellular Matrix (ECM) of the Superficial Layer of the Lamina Propria

The SLP primarily vibrates in sync with the epithelium forming a traveling wave. After injury to the vocal fold mucosa, the SLP is the most important and main site of wound healing. The tissue properties of the SLP are defined by deposition of ECM. As mentioned above, the SLP has a low content of fibrous proteins, but is rich in a variety of interstitial proteins (proteoglycans: PGs) and glycosaminoglycans (GAGs). These molecules play a key role in determining the properties of the SLP.

One of the most important molecules is hyaluronic acid (HA). HA is a mucopolysaccharide and one of the glycosaminoglycans found in the vocal fold. It is abundant in the SLP, and is thought to be a key molecule for determining the viscoelasticity of the vocal fold mucosa [7]. In general, a higher content of HA leads to an increase in tissue viscosity [8]. Rheological studies have revealed that HA has a similar viscoelasticity to the human vocal fold mucosa [9], while removal of HA alters the stiffness and viscosity of the vocal fold [10].

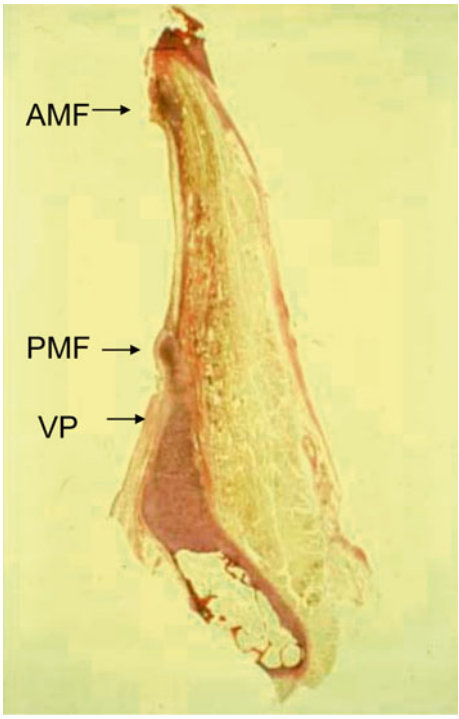

Fig. 12.3 Axial plane of the vocal fold. *AMF* anterior macula flava, *PMF* posterior macula flava, *VP* vocal process (By courtesy of Minoru Hirano)

There are many other important PGs and GAGs that contribute to the viscoelasticity of the vocal fold mucosa, these include fibronectin, decorin, biglycan, and fibromodulin. The function of these molecules is now under intensive research [11, 12].

12.1.5 Fibroblasts in the Lamina Propria

Fibroblasts in the lamina propria are primarily responsible for producing ECM. Two different kinds of fibroblasts have been reported: fibroblasts in Reinke's space (SLP) and fibroblasts in the macula flava [13]. Fibroblasts are sparse in the SLP where most of these cells are oval or spindle-shaped. Hirano et al. have found only occasional intracellular organelles such as the Golgi apparatus (GA) and rough endoplasmic reticulum (rER) in fibroblasts from the SLP, indicating lower protein-producing activity by these cells.

The macula flava is located at the anterior and posterior ends of the intermediate layer of the lamina propria (ILP) (Fig. 12.3) [14]. The anterior macula flava forms an oval mass near the anterior end of the ILP and is composed of a fine mesh of elastic fibers and fibroblasts. It is connected to the anterior commissure tendon anteriorly. The posterior macula flava is situated at the posterior end of the ILP and is connected to the vocal process. The macula flava is abundant in fibroblasts, and

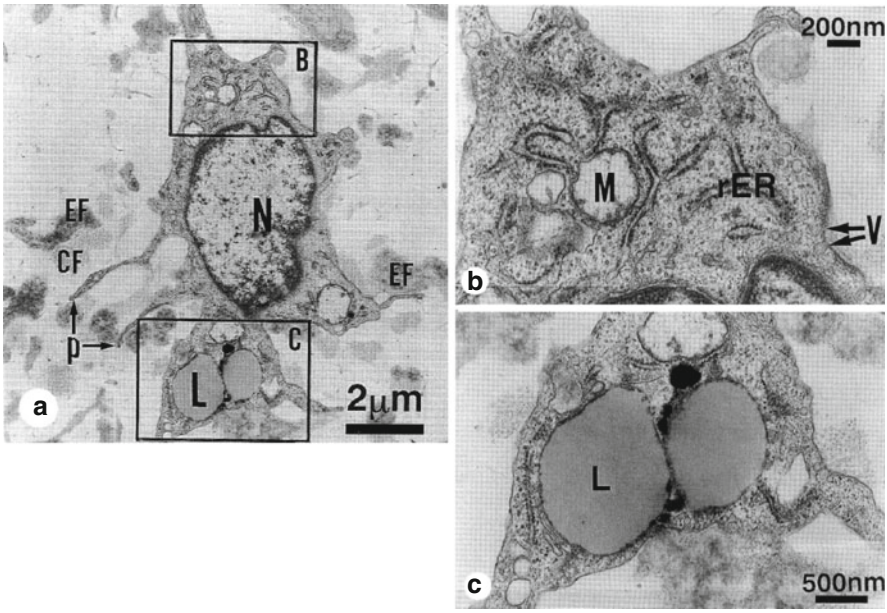

Fig. 12.4 Fibroblasts in the macula flava. (**a**) Stellate shape. *N* nucleus, *EF* elastic fiber, *CF* collagenous fiber. *L* lipid droplet, *B* and *C* indicates the figures **b** and **c**. (**b**) Intracellular organelle. *rER* rough endoplasmic reticulum, *V* vesicle, *M* mitochondria. (**c**) Lipid droplet containing vitamin A (Cited with permission [15])

these fibroblasts are predominantly stellate in shape (Fig. 12.4) [15]. GA and rER are prominent in these cells, suggesting active production of ECM proteins. Histological studies have demonstrated increased production of hyaluronic acid, collagen, and elastin around the fibroblasts. Based on these histological features, it has been suggested that the cells in the macula flava may contribute to the maintenance of the ECM in the lamina propria, although few studies have conducted functional analysis of these cells to support this hypothesis.

12.2 Alteration of the Vocal Fold

12.2.1 Vocal Fold Scar

Vocal fold scarring is a consequence of inflammation or injury to the vocal fold mucosa, which stiffens the mucosa [16] (Fig. 12.5). The scarred vocal fold has difficulty vibrating, which causes the voice to become harsh, hoarse, strained, and even aphonic. Stroboscopic examination of scarred vocal folds shows asymmetric amplitude of vibration and reduced/absent mucosal wave with glottic incompetence.

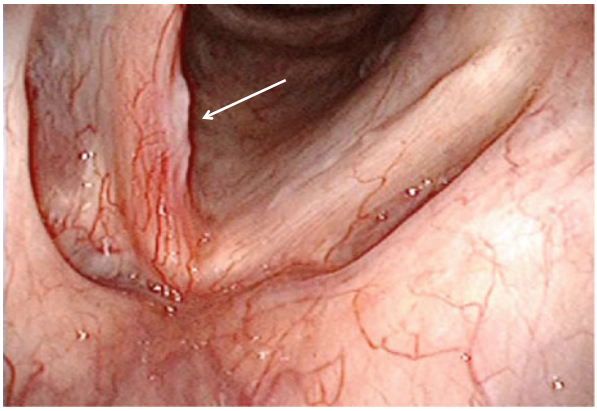

Fig. 12.5 Vocal fold scar. *Arrow* indicates scar tissue with higher stiffness

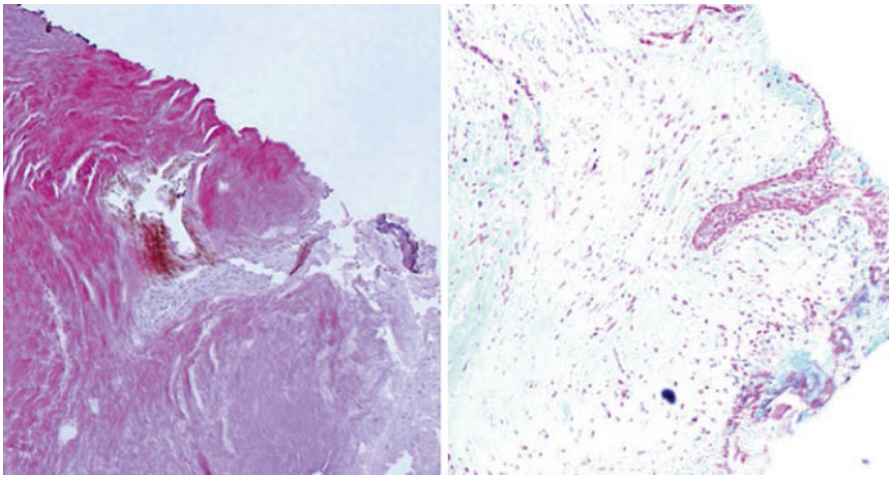

Fig. 12.6 Histology of scarred vocal fold. (*Left*) Elastica-von-Gieson stain (×4). The lamina propria is occupied with disorganized collagen (stain red) forming thick bundles. (*Right*) Alcian blue stain (×4). Few hyaluronic acid is observed

To date, there has been no optimal treatment established for restoring normal properties to the scarred vocal fold.

Histological alterations within the SLP are the main cause of stiffening of the scarred vocal fold. Histological analysis of human scarred vocal fold tissue after cordectomy shows increased and disorganized collagen deposition forming thick bundles, decreased elastic fibers and/or hyaluronic acid (HA) in the SLP [17] (Fig. 12.6). These findings cause fibrosis of the mucosa with reduced viscoelastic property. An increase in fibronectin in scarred vocal folds is thought to contribute to the increasing stiffness of the mucosa. Several animal studies using vocal fold scar models have also revealed similar histological findings [18–20]. It is clear that restoration of this deteriorated structure is key to regenerating the vocal fold.

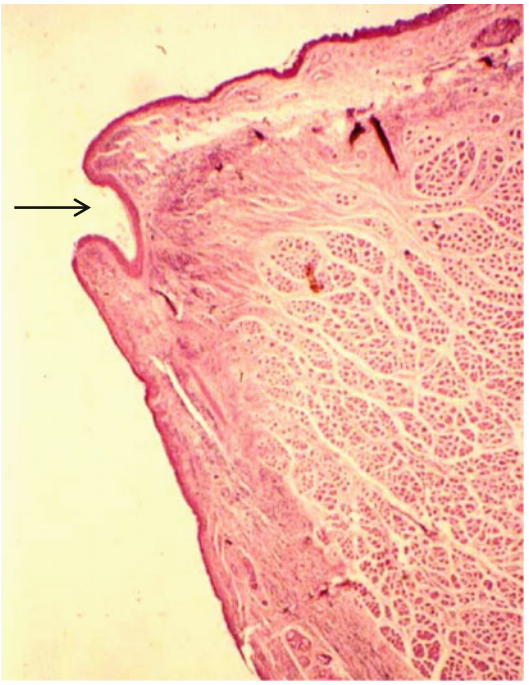

Fig. 12.7 Histology of Sulcus vocalis. Sulcus (*arrow*) is formed at the free edge of the mucosa where the epithelium is attached to the vocal ligament (By courtesy of Minoru Hirano)

12.2.2 Vocal Fold Sulcus (Sulcus Vocalis)

Vocal fold sulcus is defined as a deformation of the vocal fold mucosa with a sulcus along the mucosa; this causes a spindle-shaped glottis with bowing of the mucosa (Fig. 12.7). The sulcus causes a decreased mucosal wave, decreased amplitude of mucosal displacement, and makes the vibration aperiodic and asymmetric. This results in the voice becoming breathy, hoarse, strained, and strangled.

The etiology of sulcus formation is controversial [21]. It can occur congenitally or be acquired by inflammatory processes. The histology often shows that the epithelium is attached directly to the vocal ligament at the bottom of the sulcus, and the SLP is replaced with fibrotic tissue. This results in the mucosal wave being disturbed at the sulcus. Although several phonosurgical treatments have been attempted to improve voice, complete and consistent treatment of the deformed mucosa remains difficult.

12.2.3 Vocal Fold Atrophy

Atrophy of the vocal fold mucosa usually occurs during the aging process. With age, the vocal fold mucosa becomes thin and bowed, which causes reduced amplitude of the mucosal wave and glottic insufficiency. This results in the voice becoming hoarse and harsh. Histological analysis indicates increased deposition of

disorganized collagen and reduced elastin/HA in the SLP, causing fibrotic changes in the mucosa [22, 23]. The treatment of aged vocal folds also remains a challenge. Voice therapy, including vocal exercise, can help in some cases, and medialization procedures have been shown to have limited effects. It is thus important to regenerate the mucosa in order to treat vocal fold atrophy.

12.3 Regenerative Medicine for the Vocal Fold

12.3.1 Regeneration Targets and Strategy

Targets of regenerative medicine for the vocal fold include the disturbed vocal fold mucosa in cases such as scarring, sulcus, and atrophy. Based on the findings described above, regeneration of the vocal fold requires multiple elements including cells and ECM. The key is to restore the normal distribution of ECM in the mucosa, which requires the modulation of cell function in the vocal fold. The targets of a regenerative approach should be the cells in the vocal fold including fibroblasts, endothelial cells, epithelial cells, muscle cells, and possibly tissue-specific stem cells. Another regenerative target is the ECM of the mucosa itself.

Tissue engineering approaches involve cells, scaffolds, and regulatory factors. One of the most potent regulatory factors is growth factors that modulate cell function in terms of proliferation, migration, and production of ECM proteins. The appropriate combination of scaffold/cell/growth factor should be more effective for regenerating the vocal fold than any factor alone.

12.3.2 Scaffolding

Scaffolding strategies aim to regenerate tissue by implanting regenerative scaffold into the target organ (Fig. 12.8). Ideal regenerative scaffolds are biocompatible and biodegradable. They should also possess properties that attract an influx of cells and growth factors from surrounding tissues. A favorable interaction between scaffold and cells will enable the formation of new healthy tissue. Since a scaffold is a foreign body, it should be absorbed over time but should be retained for a long enough period to achieve the formation of new tissues. Several types of scaffolds have been studied for use in regeneration of the vocal fold.

12.3.2.1 Collagen-Gelatin Material

We have examined the feasibility of bovine-derived atelocollagen sponge (Terdermis ®, Terumo CO., Tokyo) as a scaffold for vocal fold regeneration. To first confirm biocompatibility of the scaffold, rat mesenchymal stem cells (MSCs) were cultured

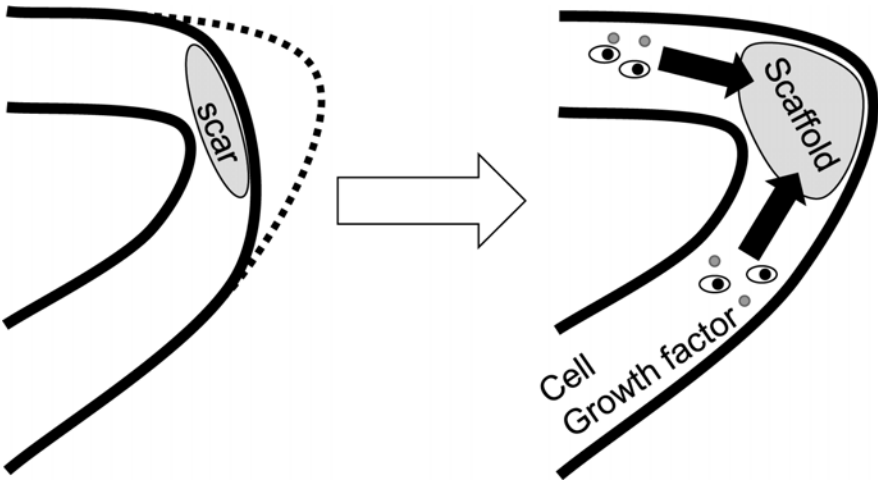

Fig. 12.8 Scheme of Scaffolding. The scar tissue is replaced with scaffold. Cells and growth factors are expected to be supplied from surrounding tissue

on the scaffold and the cells were found to survive and proliferate well, producing fibronectin [24]. A subsequent in vivo study [25] examined the effects of implanting the material into scarred vocal folds in a canine model. The results indicated that scarred canine vocal folds were softened and showed improvement of vibratory function. The histology also showed recovery of hyaluronic acid (HA) in the lamina propria of the vocal fold. These effects were strengthened by combining the scaffold with MSCs.

Based on these findings, we implanted the material (without cells) into scarred vocal folds or sulcus vocalis in six human patients [26] (Fig. 12.9). The clinical results showed gradual improvement of acoustic parameters of voice over 6 months, although individual variation was noted. While it will be important to explore a more consistent strategy, scaffolding remains an option for treating stiffened vocal folds, and may be the most feasible method that can be applied to human subjects.

Gelatin is a solubilized form of collagen, and may have potential as a scaffolding for the vocal fold. Zhang et al. [27] treated 12 sulcus vocalis patients with gelatin sponge implants coupled with fat injection. After 6 months of follow-up, improved maximum phonation time (MPT), shimmer, jitter, and noise ratio were reported. The effects of gelatin alone are not clear because of the combined use of fat injection; however, gelatin is known to adhere to growth factors. As will be described below, gelatin can act as a growth factor drug delivery system due to its unique characteristics.

12.3.2.2 Hyaluronic-Acid-Based Hydrogel

Hyaluronic acid (HA) has also received widespread attention as a candidate regenerative scaffold for the vocal fold. As mentioned above, HA is the key molecule for maintaining ideal viscoelasticity of the vocal fold mucosa. A group in Boston,

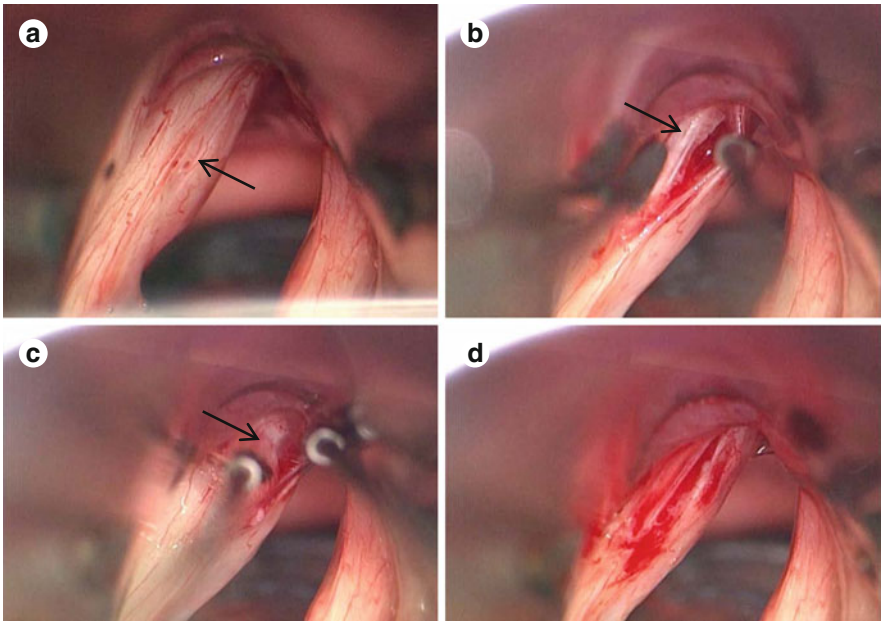

Fig. 12.9 Regenerative surgery consisting of superficial cordotomy and dissection of scar (**b**), implant of scaffold (**c**). (**a**) *Arrow* indicates sulcus. (**b**) *Arrow* indicates the vocal ligament. (**c**) *Arrow* indicates scaffold. (**d**) Indicates post operative status

Massachusetts has developed an HA-based "microgel" as an injectable material for the vocal fold [28]. An in vitro study with fibroblast cultures showed low or no toxicity. Mechanical measurements using a torsional wave apparatus indicated that this HA-based microgel exhibited elastic moduli similar to the lamina propria of the vocal fold at frequencies close to the range of human phonation. This study suggested that the microgel may be useful for regeneration of the vocal fold lamina propria.

A Madison, Wisconsin group has also developed HA hydrogels [29]. Chemically modified HA-gelatin hydrogels were implanted into the injured vocal folds of rabbits, and gene expression analysis of the treated vocal folds indicated upregulated levels of mRNA for procollagen type I, fibronectin, TGF-beta1, fibromodulin, HA-Synthase 2, and hyaluronidase 2. The treated vocal folds also exhibited significantly improved tissue elasticity and viscosity.

One concern of these HA-based materials is that they are artificial forms of HA and not the native version of HA that endogenously exists within the vocal fold. Unfortunately, the characteristics of native HA have not been described. It is well known that the biological behavior of HA depends on its molecular weight. Munoz-Pinto et al. [30] showed that vocal fold fibroblasts cultured in HA hydrogels showed differences in gene expression according to the molecular weight of the HA. It will be important to explore the ideal molecular weight of HA if these types of scaffolds are to be used.

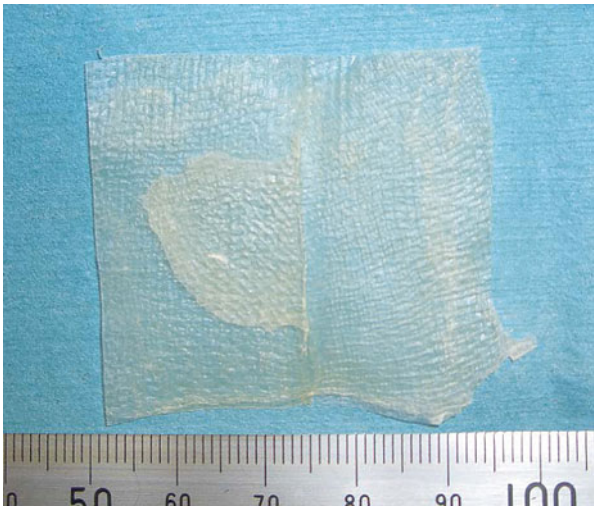

Fig. 12.10 Urinary-bladder-derived decellularized scaffold

12.3.2.3 Decellularized ECM ("Bioscaffold")

Decellularized ECM is a relatively new material that has promising potential as a regenerative scaffold. Decellularization can be achieved by physical, chemical, and enzymatic processes [31]. Many recent reports have demonstrated successful decellularization of whole organs including heart, lung, kidney, liver, bowel, and skeletal muscle [32]. The technique allows production of complex 3D ECM bioscaffolds with preservation of intrinsic vascular networks as well as ECM components. Recellularization is then possible with autologous stem cells. Xu et al. [33] developed an acellular xenogeneic ECM scaffold derived from bovine vocal fold lamina propria, and confirmed biocompatibility of the scaffold with human vocal fold fibroblasts.

Badylak et al. [34] developed a porcine urinary bladder-derived ECM scaffold which contains several growth factors as well as ECM components (Fig. 12.10). We have applied this material to hemilaryngectomy defects in canine larynx, and found simultaneous regeneration of the cartilage, muscle, and the vocal fold mucosa [35] (Fig. 12.11). The primary regeneration of the mucosa was completed as quickly as 2 weeks after treatment. The vibratory property of the mucosa varied by individual, but the scaffold represents a promising material for laryngeal regeneration.

The development of decellularized whole larynx has also been attempted. Baiguera et al. [36] made decellularized whole human larynx using DNAse and sodium deoxycholate, and confirmed decellularization of the cartilage, false vocal fold, and vocal fold. Preservation of the mechanical properties of the cartilage was also noted. In the future, this type of technique may enable regeneration of the whole larynx after total laryngectomy.

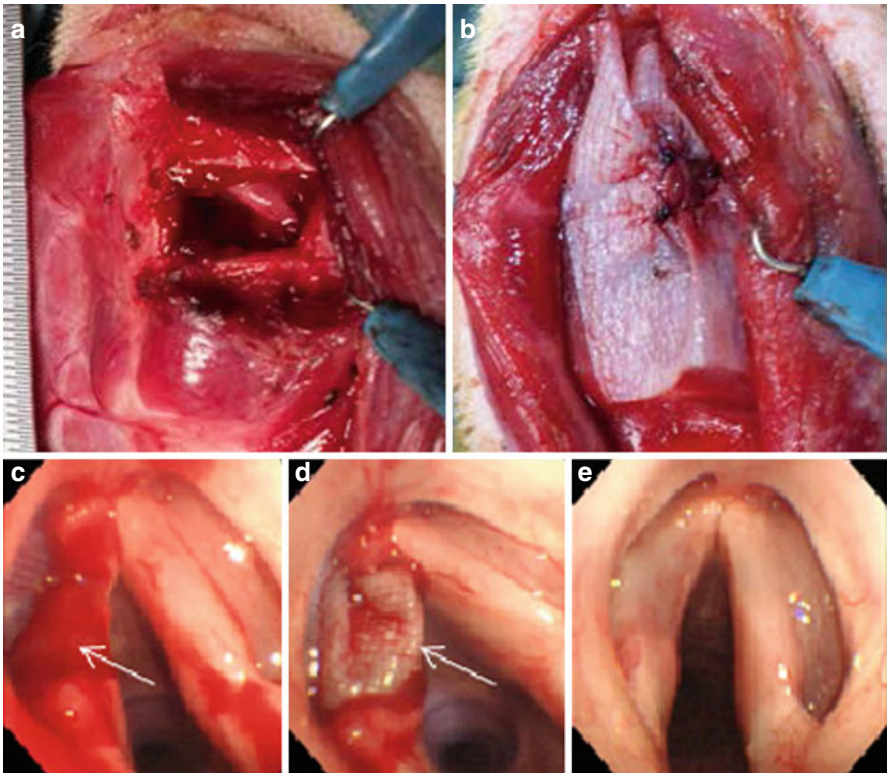

Fig. 12.11 Reconstruction of hemilaryngectomy with decelluralized ECM scaffold. (**a**) Hemilaryngectomy defect of canine larynx. (**b**) Implant of scaffold. (**c**) Endoscopic findings of hemilaryngectomy. *Arrow* Defect after hemilaryngectomy. (**d**) Implant (*arrow*) of scaffold. (**e**) Regeneration of the hemiglottis

12.3.3 Cell Therapy

Cell therapy is the most effective tool for tissue regeneration. Cells implanted into the vocal fold mucosa can proliferate, migrate, and produce ECM proteins, which can lead to recreation of the histological architecture of the mucosa. Stem cells are expected to not only differentiate into several kinds of cells, but also to work in a paracrine manner by producing several growth factors and cytokines. Several types of cells have been studied as candidates for use in vocal fold regeneration.

12.3.3.1 Fibroblasts

We were first to report the use of vocal fold fibroblasts to restore scarred canine vocal folds [37]. The results, however, were disappointing because the vocal folds, treated by injection of autologous vocal fold fibroblasts, remained severely scarred. A group at UCLA also conducted fibroblast injections into injured canine vocal

folds [38]. They used autologous buccal mucosa fibroblasts and the results were encouraging, showing improved mucosal wave of the vocal folds. The key question is how to control ECM production by the fibroblasts that are implanted into the vocal fold. Moreover, the consistent and appropriate application of mature cells remains difficult, thus many researchers focus more attention on immature cells including several types of stem cells.

12.3.3.2 Bone-Marrow-Derived Mesenchymal Stem Cells (MSCs)

It is well known that bone marrow contains mesenchymal stem cells as well as hapatopoietic stem cells. MSCs are multipotent and can differentiate into several tissues including nerve, muscle, cartilage, tendon, adipose, and cardiac muscle. It has also been shown that MSCs contribute to wound healing [39]. A population of MSCs circulates in the peripheral vessels and upon tissue damage, these MSCs migrate and home to the injury site to repair the tissue.

Another important aspect of MSCs is their reported ability to produce several kinds of growth factors and ECM such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and collagen [40]. Ohno et al. revealed that MSCs have significantly higher levels of hyaluronic acid synthase (HAS) and MMP-1 mRNA expression than vocal fold fibroblasts, which led to the hypothesis that MSC implantation would be effective for vocal fold regeneration in cases of scarring, sulcus, and atrophy [41].

Injection of MSCs into injured tissue has shown promising results. It was reported that MSCs injected around wound sites caused enhanced re-epithelialization, cellularity, and angiogenesis with increased levels of VEGF and aniopoietin-1 [42]. Another study also showed that the addition of MSCs increased collagen matrix and induced wound healing angiogenesis during early stages of wound healing [43]. Kanemaru et al. developed a cell culture system for bone-marrow-derived MSCs and treated injured canine vocal folds with an injection of autologous MSCs [44]. The results were encouraging, demonstrating better tissue recovery of the injured vocal folds. The MSCs were positive for mesenchymal stem cell markers, including CD29, CD44, CD49e, and Sca-1. Moreover, implanted MSCs in nude rat vocal folds demonstrated positive expression for keratin and desmin, markers of epithelial tissue and muscle, respectively [45]. It was further confirmed that injected MSCs are multipotent and can differentiate into several tissues including epithelium, muscle, and mesenchyme.

Hertegard et al. also treated scarred vocal folds by injection of human MSCs [46]. They found lower viscosity and elastic modulus of the treated vocal folds compared to untreated scar tissue, though the viscoelasticity did not reach normal levels. Histological analysis showed less collagen type I and in this study, the injected MSCs persisted for 4 weeks.

We performed an additional study using a combination of collagen scaffold and MSCs for the treatment of canine scarred vocal folds [25]. The results indicated better regeneration of the vocal folds treated with the combination therapy than those treated with scaffold alone.

Since MSCs are a source of autologous cells, clinical application is feasible and warranted.

12.3.3.3 Adipose-Derived Mesenchymal Stem Cells (ASCs)

It is well known that fat tissue includes mesenchymal stem cells called ASCs. ASCs have similar biological activity and the multipotent ability to differentiate into several kinds of cells as MSCs. Cultured ASCs were shown to produce HGF, IGF, VEGF, and other cytokines, and are thought to be suitable for vocal fold regeneration [47].

Lee et al. examined the effects of ASC injection into injured canine vocal folds and found the continued presence of cells within the tissue after 6 months [48]. The treated vocal folds also appeared to have better morphology. It was suggested that remaining ASCs might work in a paracrine manner for regeneration of the vocal fold. Xu et al. also treated scarred vocal folds of rabbits with ASC implantation [49]. The results demonstrated better regeneration of the vocal folds as compared to those treated with scaffolding only.

There has been no comparative study between MSCs and ASCs in terms of their effects on vocal fold regeneration, although the effects are thought to be similar. Thus, ASCs are also an appropriate tool for restoration of scarred and/or atrophied vocal folds.

12.3.3.4 Tissue-Specific Stem Cells

It has become well known that many tissues harbor their own specific stem cell population, which resides permanently in most organs and can generate mature cells required for organ function. These tissue-specific stem cells have been identified in the skin, intestine, eye, brain, muscle, and other tissues. They are located in so-called niches, similar to the bulge cell population found in skin. However, it has not been determined if the vocal fold has a tissue-specific stem cell population.

We attempted to identify side population (SP) cells in the vocal fold mucosa using FACS with Hoechst stain, and found that SP cells comprised approximately 0.2% of the cells in the human vocal fold mucosa [50]. SP cells are regarded to have abundant stem cells, and were located at or around the epithelium and lamina propria of the vocal fold. These findings indicate the possible presence of tissue-specific vocal fold stem cells.

In a subsequent in vivo study, rat vocal folds were injured and expression of SP cells was examined during wound healing [51]. The results showed the appearance of SP cells during the early phase of wound healing, within 1 week, after which point the SP cells disappeared. It was suggested that SP cells may contribute to wound healing or possible regeneration of injured vocal folds during the early stages after injury.

SP cells are regarded as a population consisting of several cells that have immature features, thus it is difficult to identify pure tissue stem cells. Future research is

needed to confirm the presence of stem cells within the vocal fold and, once identified, they are likely to be an effective tool for vocal fold regeneration.

12.3.3.5 Embryonic Stem (ES) Cells /Induced Pluripotent Stem (iPS) Cell

ES cells are the origin of every cell in the body, and have the most potential to regenerate any organ. Cedervall et al. examined the regenerative effects of ES cell injection into scarred rabbit vocal folds, and demonstrated significantly improved viscoelasticity of the injected vocal fold mucosa [52]. The injected ES cells were found to remain within the tissue for 1 month. However, there are several problems associated with treatment using ES cells, such as ethical considerations, immune response, and tumor formation; thus, research into ES cells has not progressed in the field of laryngology.

The iPS cell represents a breakthrough in stem cell technology and can substitute for the use of ES cells. These cells can be developed using autologous tissues, avoiding the ethical issues and immune response. Although numerous studies are underway to develop regenerative strategies using iPS cells, there has been no report of their use in laryngology. The biggest concern with their use is the possibility of tumor formation. Imaizumi et al. first reported on the implantation of iPS cells into tracheal defects in rats, and showed cartilage regeneration in two of five rats, although teratoma formation occurred in many cases [53]. Investigators in several fields are currently working to reduce the possibility of tumor formation after iPS cell implantation.

12.3.4 Growth Factor Therapy

Growth factor therapy is another effective strategy in tissue engineering. Exogenous growth factors are thought to stimulate cells and modulate their function by changing their phenotype. Growth factor treatment jump starts the healing process and induces tissue regeneration. Therefore, simple application of a growth factor solution should have positive regenerative effects, but for refractory cases, repeated application or a drug delivery system is required to strengthen these effects.

12.3.4.1 Hepatocyte Growth Factor (HGF)

We have extensively researched the use of hepatocyte growth factor (HGF) for regeneration of the vocal fold due to its strong anti-fibrotic activity. HGF was first identified as a growth factor for hepatocytes, but subsequent studies revealed that HGF is expressed in many organs and contributes to organogenesis, angiogenesis, and regeneration [54].

We have also confirmed that HGF is produced by vocal fold fibroblasts, and that the vocal folds express the HGF receptor c-Met [55]. Through in vitro

studies using cultured vocal fold fibroblasts, we demonstrated that HGF stimulates production of hyaluronic acid (HA) and suppresses collagen synthesis from fibroblasts, which are regarded as positive effects for the prevention or treatment of vocal fold scarring [56]. Krishna et al. also reported increased production of HAS from rabbit vocal fold fibroblasts with application of HGF [57]. Luo et al. reported that HGF stimulated production of HA and elastin and suppressed collagen deposition in three-dimensional cultures of fibroblasts in hydrogel [58]. Kishimoto et al. further confirmed that exogenous HGF stimulates production of endogenous HGF from vocal fold cells, suggesting an autocrine loop for HGF signaling [59].

Subsequent animal studies using rabbits and dogs showed remarkable regenerative effects of HGF in the prevention and treatment of scarred vocal folds. In a rabbit study, HGF was administered just after stripping the vocal folds, and the results indicated better wound healing and regeneration in the treated folds [60]. Viscosity and stiffness of the vocal folds were also significantly improved compared to nontreated scarred vocal folds. In a canine study, HGF was injected into the scarred vocal folds 1 month after initial injury, and the effects were examined 6 months after treatment. The results indicated better tissue properties and improved histological architecture of the vocal fold mucosa, with restored levels of HA and abundant well-organized collagen [37]. We also developed a novel drug delivery system for HGF using gelatin hydrogel, which enabled the controlled-released of HGF in vivo over 2 weeks. This drug delivery system has proven to be useful for treatment of not only acute scarring but also chronic scarring of the vocal fold in the same canine model of wound healing described above [61]. HGF has also been shown to be effective for aged vocal fold atrophy. Ohno et al. locally applied HGF to aged rat vocal folds and revealed up-regulated expression of mRNA for HAS and MMP in the treated vocal folds, as well as improved histology of the aged vocal folds with increased HA and decreased collagen [62]. Suehiro et al. explored the optimal dose of HGF for the treatment of aged vocal fold atrophy in rats. They concluded the most desirable biochemical effects of HGF occurred at a concentration of 100 ng/10 uL, based on the ability to most effectively upregulate expression of HAS-2 and MMP-9 mRNA and decrease expression of collagen type I [63].

While HGF has been clearly shown to be an effective tool for vocal fold regeneration in cases of scarring, sulcus, and atrophy, unfortunately no commercial product has yet been developed for human use. However, 5 amino acid-deleted type HGF (dHGF) was recently developed using good manufacturing practices (GMP) and is compatible for use in humans [64]. This form of dHGF has been shown to have similar biological activity to full-length HGF. We conducted an experiment to examine the effects of both types of HGF on restoration of scarred vocal folds in a canine model, and confirmed that both forms of HGF have similar regenerative effects on the vocal fold in terms of vibratory function and histological aspects [65] (Fig. 12.12). A clinical trial is expected using the GMP-compatible HGF product in the future.

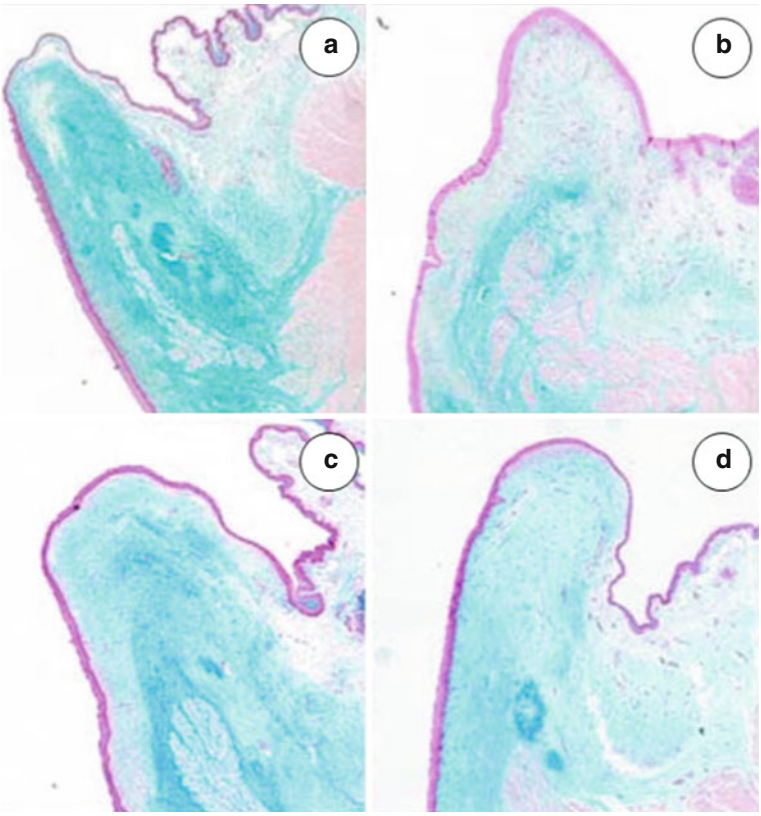

Fig. 12.12 Histology of canine vocal folds treated with growth factors. (**a**) Normal, (**b**) sham scar, (**c**) full HGF, (**d**) deleted type HGF. Hyaluronic acid (stained blue) is diminished in sham, while it is restored in both full and deleted-type HGFs

12.3.4.2 Basic Fibroblast Growth Factor (bFGF)

We have examined the therapeutic potential of bFGF for regeneration of the vocal fold. Basic FGF is a stimulant of fibroblasts and induces cell migration and proliferation; it also contributes to angiogenesis and epithelialization. Several in vitro studies have demonstrated that bFGF stimulates HA synthesis and suppresses expression of the collagen gene [66, 67].

We have reported that bFGF stimulates HA production in aged rat vocal fold fibroblasts, and subsequently confirmed its regenerative effects by injecting bFGF into aged rat vocal folds [68, 69]. Histological examination indicated that bFGF-treated vocal folds showed recovery of HA distribution in the lamina propria with reduced collagen deposition (Fig. 12.13). Ohno et al. further examined the effects on the genetic level using PCR [70]. They found that bFGF up-regulated hyaluronic acid synthase (HAS) 2 and 3, as well as MMP2 in aged rat vocal folds. Based on this basic research, we set up clinical trials for treatment of aged vocal fold atrophy with

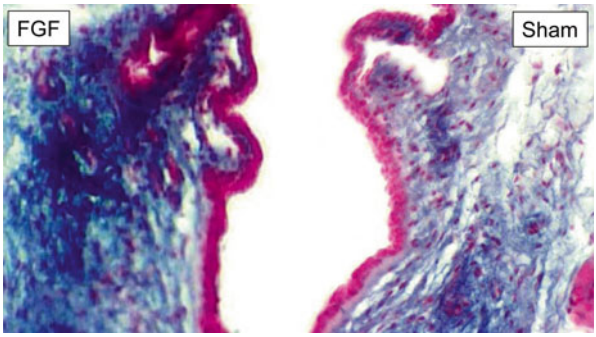

Fig. 12.13 Histology of rat vocal folds treated with basic fibroblast growth factor. Basic FGF improved deposition of hyaluronic acid

local injection of the bFGF product Fibrast ® (Kaken Co. Tokyo). The first case was reported in 2008 with excellent results indicating improved vibratory properties with better voice after administration of bFGF [71] (Fig. 12.14). When ten cases were analyzed, the data showed improved phonatory function such as MPT, jitter, shimmer, and noise parameter. Mucosal wave amplitude of the vocal folds was also significantly improved during the 1 year period after treatment [72].

We also attempted to treat vocal fold scarring with bFGF in a canine study. Repeated injection of bFGF solution showed good restoration of scarred vocal folds in terms of mucosal vibration and histological findings [73]. In the clinical setting, bFGF has been applied to human cases of vocal fold scar or sulcus by means of injection of bFGF solution or regenerative surgery using bFGF. Regenerative surgery consists of elevation of a microflap, dissection of scar tissues in the lamina propria, and implantation of gelatin scaffold soaked in bFGF solution [72] (Fig. 12.9). The results indicated significant improvement in MPT, GRBAS scale, and voice handicap index −10 with improved vibratory function (Fig. 12.15). Although improvement of acoustic parameters was not consistent across cases, regenerative treatment has clearly been shown to be feasible using bFGF.

In conclusion, the therapeutic effects of both HGF and bFGF have been confirmed for either scarred or atrophied vocal folds.

12.3.4.3 Comparison of HGF Versus Basic FGF

There has been no direct study comparing the regenerative effects of HGF and basic FGF on the vocal fold. Figure 12.16 shows the analysis of normalized mucosal wave amplitude (NMWA) in canine studies [65, 73] in which 1-month-old scarring was treated by injection of saline (sham), full HGF, deleted type HGF (dHGF), and bFGF. NMWA was significantly improved by injection of either form of HGF or bFGF when compared to sham. Both forms of HGF seemed to be superior to bFGF, although the difference did not reach statistical significance. These studies were not performed simultaneously, however, thus the results only suggest a similarity of effects of each growth factor.

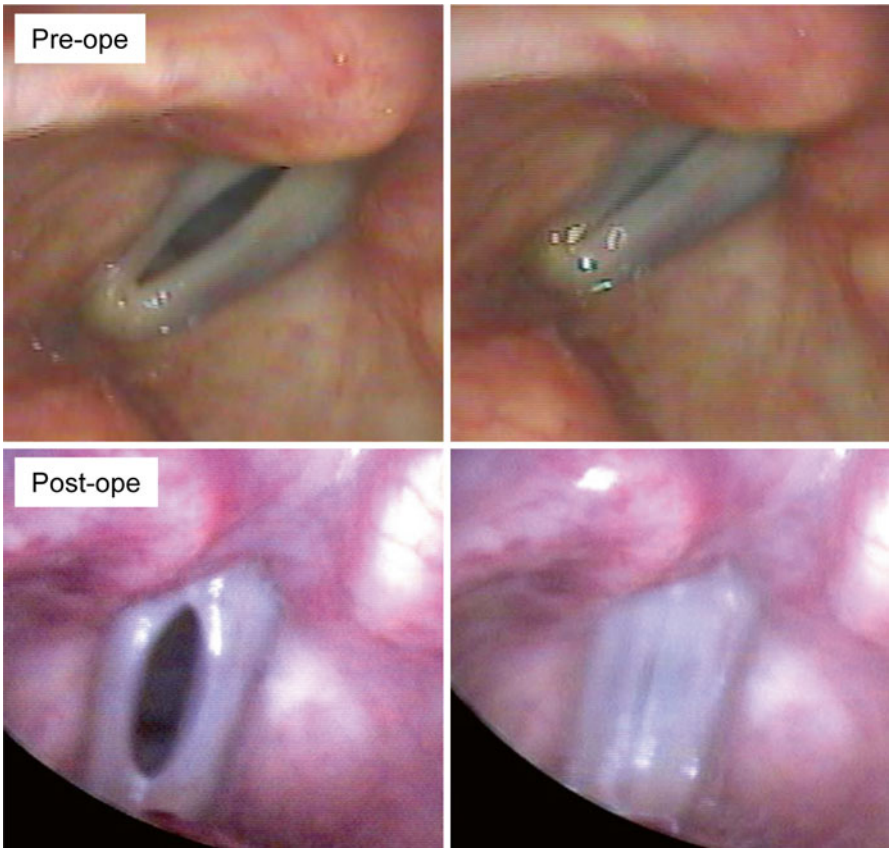

Fig. 12.14 Clinical case of aged vocal fold atrophy treated with bFGF injection. After injection, the vocal fold closure becomes complete, and the mucosal wave becomes much wider

12.4 Future Directions

Regeneration of the vocal fold mucosa remains challenging owing to the sophisticated layer structure of the vocal fold and its vibratory properties which are difficult to restore. Growth factors, with or without the addition of a scaffold, are feasible and effective for clinical application. The main issue is how to strengthen the effects and improve consistency across individuals. A novel drug delivery system (DDS) should be developed to improve this aspect. Gelatin may be the best scaffold for growth factors as a DDS carrier, but other more optimal materials should be explored. Another issue is the determination of the most suitable growth factor for vocal fold regeneration. To date, bFGF and HGF have proven to be the best candidates, but it is not yet known which is better or the advantages and disadvantages of each growth factor compared to the other. A comparative study among these growth factors is thus warranted in the future.

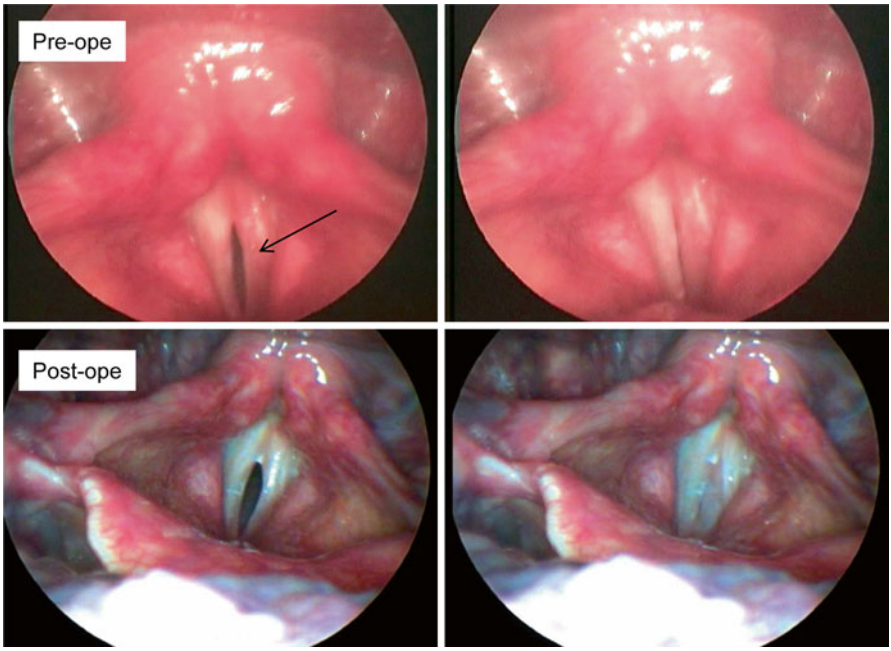

Fig. 12.15 Clinical case of scarred vocal fold treated by regenerative surgery with bFGF. Preoperative findings showed reduction of mucosal wave with glottic incompetence particularly on the left (indicated by *arrow*). Postoperative findings indicates better mucosal vibration with complete glottal closure

Cell therapy may be the strongest tool to regenerate the vocal fold in cases of scarring, sulcus, and atrophy. Several cell sources are available including MSCs, ASCs, or even iPS cells, although it is also not known which is best, or what advantage each cell source has over the other. Clinical trials are thus needed to confirm the safety and effectiveness of each cell source.

Decellularized ECM scaffolding may have great potential for regenerating not only the vocal fold but also other tissues of the larynx including the cartilage and muscle. This strategy may contribute to reconstruction after total or partial laryngectomy. Ideal methods to decellularize the larynx should be explored in terms of effectiveness, safety, and possible tissue damage.

Given that the larynx is the most important organ for vocal communication output, it is essential to develop regenerative medicine for the larynx.

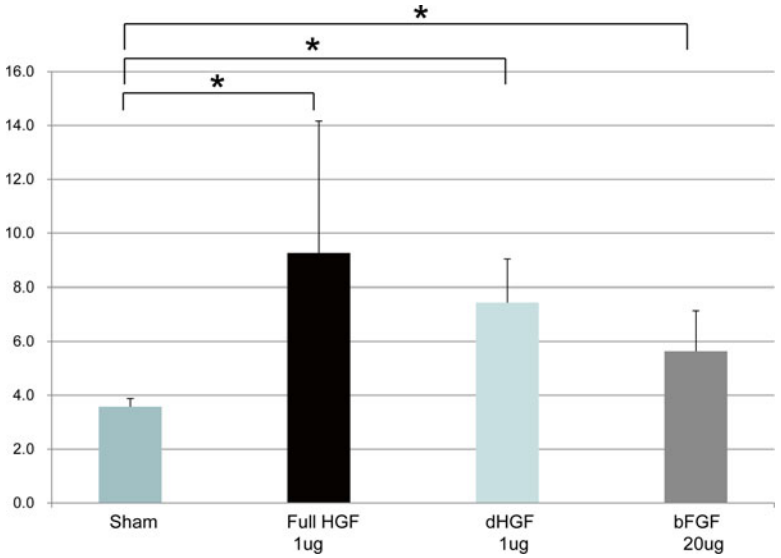

Fig. 12.16 Comparison of mucosal wave amplitude. Full HGF, deleted-type HGF, basic FGF significantly improves mucosal wave amplitude as compared to sham scar. Both HGFs seem to be superior to bFGF, though the difference does not reach statistical significance. *Asterisk* means statistical significance <0.05

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Part VI
Development and Regeneration of the Head and Neck

Chapter 13
Cranial Bone Regeneration

Hiroo Umeda

Abstract Cranial bone regeneration is challenging, because flat bones, including the skull, are more difficult to regenerate than tubular bones. However, promotion of cranial bone regeneration would directly benefit patients, who would be spared cosmetic handicaps including postoperative facial deformities commonly associated with the current techniques of bone reconstruction. We studied cranial bone regeneration and confirmed that collagen-coated beta-tricalcium phosphate (b-TCP) is a suitable raw material for regeneration of cranial bone and that bone marrow-derived stromal cells (BSCs) have the potential to promote bone regeneration. However, excessive fibrous tissue intrusion into the regenerative site or dislocation of the bone substitute material occasionally interrupted bone regeneration. To avoid such problems, we next assessed calcium alginate (CA) membrane, which is useful for guided bone regeneration (GBR), to investigate whether this material maintains the bone regenerative space. CA membrane prevents excessive fibrous tissue intrusion and/or dislocation of a bone scaffold. However, we found that CA membrane did not always accelerate cranial bone regeneration. Our results demonstrated that a supplementary supply of vessels or regulatory factors from superficial structures may be indispensable in cranial bone regeneration.

Keywords Cranial bone regeneration • *In situ* tissue engineering • Beta-tricalcium phosphate • Collagen • Bone marrow-derived stromal cells • Calcium alginate membrane

13.1 Introduction

Remarkable progress has been made recently in regenerative medicine. With regard to bone regeneration, there are many reports describing the efficacy of various scaffolds [1–6], some of which are used clinically for the repair of tubular bones. In contrast, flat bones, including those of the skull, are more difficult to regenerate than

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tubular bones because of their lack of bone marrow. Conventional repair of the skull has been performed by attaching a bone flap to the surrounding bone with artificial devices such as metal plates or meshes. These repairs cause deformation of the reconstructed site after surgery and also have the disadvantage of creating imaging artifacts in computed tomography (CT), magnetic resonance imaging (MRI), and radiography. If high-quality flat bones could be generated by tissue engineering techniques, otorhinolaryngologists and head and neck surgeons would be able to repair defects and deformities caused by surgical excisions, traumas, and congenital diseases. This approach would directly benefit patients, who would be spared cosmetic handicaps including the common postoperative facial deformities associated with the current techniques of bone reconstruction. We therefore attempted to regenerate cranial bone using an *in situ* tissue engineering technique.

13.2 About *In Situ* Tissue Engineering

According to tissue-engineering principles, tissues and organs can be regenerated by manipulating three elements: cells, scaffolds, and regulatory factors [7–9]. The concept of *in situ* tissue engineering in particular means to bring tissue regeneration *in vivo* by making use of the curative power of nature. Thus, all three of the important factors are not always required to regenerate tissues if an adequate environment for tissue regeneration can be provided *in vivo*. For instance, myringoplasty using autologous fascia is applicable to this concept even though only one of the three factors is provided. The transplanted fascia has the role of scaffold, while the surrounding intact epithelia of the eardrum provide both cells and regulatory factors by scratching and removing the old edge of the epithelia. Cases when atelocollagen is used as a scaffold, or when basic fibroblast growth factor (b-FGF) is added to the site for eardrum regeneration, all fit into this concept [10].

13.3 Current Situation of Cranial Bone Surgeries

In most cases, cranial bone reconstructions have been performed by attaching bone grafts to the surrounding bone using artificial devices such as titanium plates or stainless steel wire. Because reconstructive surgery alone results in no bone regeneration around the bone graft, any gap between the skull and the graft is gradually filled in with fibrous tissue. As a result, gradual deformations of the reconstructed site after surgery could cause cosmetic handicaps for patients. To avoid these deficiencies, bone grafts can be directly attached to the skin flap on occasion, but the procedure is not common because of its difficulty and complexity. Even in cases where this method has been performed, the gaps made by the drill or saw still remain and present the same difficulties regarding regeneration. In addition, the presence of titanium plates or stainless steel wire interferes with imaging by CT, MRI, and

x-ray. The root cause of these problems could be due to the fact that the cranium is made up of flat bone. Flat bone contains little cancellous bone, in contrast to long bone. It therefore contains little bone marrow, and thus provides poor conditions for bone regeneration.

13.4 Cranial Bone Tissue Engineering

13.4.1 Three Important Factors for Skull Tissue Engineering

13.4.1.1 Scaffold

If a critical size defect in the skull does not begin the healing process, it is gradually replaced by fibrous tissue and eventually becomes a permanent scar which will never change into bone. Osteoblasts are the cells that form bone; therefore, it is important to create a suitable environment for the cells to generate new bone. In the concept of *in situ* tissue engineering, the most important factor is the scaffold for cell proliferation and differentiation. We adopted a bone substitute material that is currently clinically applied only in long bone.

Since the mid-1980s, the bone substitute materials clinically applied in clinical use are mainly calcium phosphate ceramics: hydroxyapatite and beta-tricalcium phosphate (b-TCP). Both materials have osteoconductive activity and are mainly used in orthopedic and maxillofacial surgery. Although hydroxyapatite is seldom absorbed *in vivo*, b-TCP is absorbed within 6–18 months and becomes the raw material of bone. b-TCP has many interconnected pores. The macroscopic pore diameter is 100–400 μm, and the microscopic pore size is less than 5 μm. The initial strength of b-TCP is weaker than that of hydroxyapatite [11], but a b-TCP transplanted site eventually regains ideal strength because it is absorbed and converted to native bone. Recently, a calcium phosphate bone substitute material called octacalcium phosphate has also gained attention [12, 13]. In our studies, we chose b-TCP because it was the only absorbable bone substitute material available at that time. b-TCP itself, however, has insufficient ability to regenerate cranial bone, and is not authorized for clinical cranial bone regeneration. We, therefore, utilized b-TCP with a collagen coating.

Collagen is also an important component of bone. Type I collagen is the most abundant matrix protein, providing a structural framework for connective tissues and playing a central role in the temporal cascade of events leading to the formation of new bone from progenitors [14, 15]. We expected that collagen-coated b-TCP would provide better osteoconductive activity than innate b-TCP alone.

Bone putty, which is made by drilling into the skull and mixing the shavings with fibrin glue, is often used clinically and eventually forms a bone-like tissue. This material is useful but not enough to fill a large defect. Therefore, the autologous bone putty was mixed with a composite scaffold and used as a bone substitute material. We were able to identify bone regeneration associated with the bone substitute

material in the study described later. The extent of regeneration was very restricted, so the setting for bone regeneration needed further investigation.

13.4.1.2 Cells

In our first experiment, we used b-TCP alone without any cells, but the result reflected the lack of cells at the regenerative site because the skull contains little bone marrow. We therefore added additional cells in our later studies. Bone is of mesodermal origin. We utilized bone marrow-derived stromal cells (BSCs), which are easily accessible by bone marrow aspiration and constitute a rich source of mesenchymal stem cells (MSCs) [16]. The effectiveness of MSCs for bone regeneration has been reported previously [6, 17]. MSCs are the origin of all mesodermal tissues, including bone, cartilage, muscle, and adipose tissue. MSCs can be obtained from autologous bone marrow; consequently, there are fewer concerns of immunologic rejection. Furthermore, there are fewer ethical issues associated with the use of MSCs compared with embryonic stem cells which are obtained from fertilized eggs. Thus, BSCs, as a source of MSCs, are a suitable cell source for bone regeneration. Based on the concept that cells are an essential requirement of *in situ* tissue engineering, we added BSCs without any induction of differentiation.

13.4.1.3 Regulatory Factors

The efficacy of various regulatory factors, for example, b-FGF or bone morphogenetic protein-2 (BMP-2) has been reported for bone regeneration. Recent reports showed that conditioned medium from mesenchymal stem cells, which contains insulin-like growth factor I (IGF-1), vascular endothelial growth factor (VEGF), transforming growth factor-beta 1 (TGF-beta 1), and hepatocyte growth factor (HGF), encourages bone regeneration [18]. In our study, no regulatory factors were added because growth factors are thought to be released spontaneously *in vivo*. Based on the results of recent studies, cell transplantation itself appears to stimulate the release of regulatory factors indirectly at the implantation site.

13.4.2 Guided Bone Regeneration (GBR)

Guided bone regeneration (GBR) does not itself create regenerated tissue but assists regeneration by providing a suitable environment for regeneration. GBR was first mentioned in 1959 by Hurley, who used the concept in a spinal fusion model [19]. Currently, this concept is widely recognized and used in oral dental surgery. The objective of GBR is to promote bone formation by offering a suitable environment for bone regeneration using a barrier membrane. A barrier membrane is designed to maintain the regenerative space [20, 21] and to prevent fibrous tissue intrusion [21, 22] and

infection of the bone defect [23]. Barrier membranes are categorized into two types. One is a nonabsorbable membrane, made of materials including expanded polytetrafluoroethylene (E-PTFE) and sheet-type titanium. The other is an absorbable membrane, types of which include polylactic acid (PLA), polyglycolic acid (PGA), and collagen. Calcium alginate (CA) is also reported to be an effective absorbable material for GBR and to be superior to collagen as a GBR membrane [24, 25]. CA, which is abundant in brown algae, is biodegradable, safe, and cheap. It is widely used as a food additive, such as a thickening agent for soups, jellies, and ice cream, and in the healthcare industry for antacid preparations and burn dressings [26].

13.4.3 Our Cranial Bone Regeneration Study

We carried out three experiments on skull regeneration using beagle dogs. The second and last experiments were designed to resolve the new problems brought to light in the earlier experiments. The aim of the first experiment was to examine the tissue response and new bone formation induced by b-TCP, collagen, and autologous bone fragments with fibrin glue implanted into a cranial bone defect. The second one was to investigate whether BSCs with the scaffolds mentioned above could promote regeneration of cranial bone after 3 and 6 months. The final experiment aimed to examine the usefulness of a CA membrane and to investigate whether this material maintains the bone regenerative space.

13.4.3.1 First Experiment [3]

A 2×2 cm square bone flap was created in each dog (Fig. 13.1). The cranial bone defect was closed by replacing the free bone flap back in position, and the remaining fissure and burr holes were filled with a mixture of collagen-coated b-TCP and bone putty with fibrin glue, or left unfilled, in group 1-II or 1-I, respectively (Fig. 13.1a, b). Bone regeneration was evaluated only 3 months after treatment with 3D-CT and histologic examinations of the operative regions. In group 1-II, although a part of the implanted b-TCP still remained, new bone formation was observed at the reconstructed site. However, bone regeneration was limited to a small area.

13.4.3.2 Second Experiment [27]

A bone flap was created in the same manner. Dogs were divided into three groups. In group 2-I, the bone defect was closed by replacing the original free bone flap without filling the residual gap (Fig. 13.1a). In group 2-II, the gap was filled with the bone substitute material (BSM): collagen-coated b-TCP combined with autologous bone fragments (Fig. 13.1b). In group 2-III, autologous BSCs combined with BSM were used to fill the gap (Fig. 13.1c). The effectiveness of treatment was evaluated

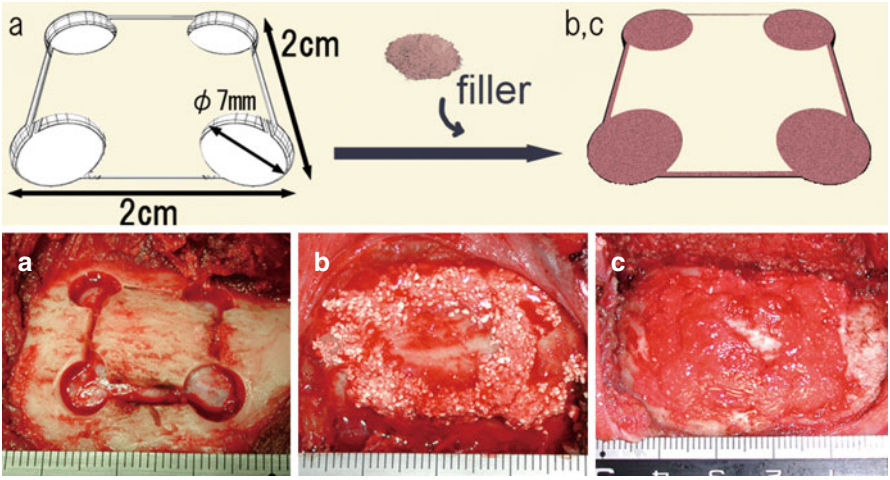

Fig. 13.1 Design of the operation and intraoperative photographs. Group 2-I (**a**), group 2-II (**b**), and group 2-III (**c**)

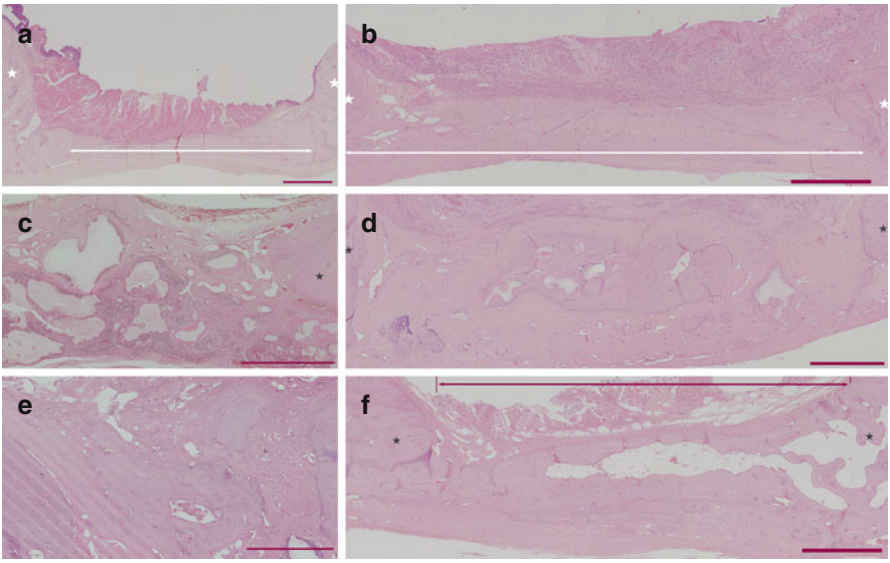

Fig. 13.2 Histology of group 2-I (**a**, **b**), 2-II (**c**, **d**), and 2-III (**e**, **f**). Three-month model (**a**, **c**, **e**). Six-month model (**b**, **d**, **f**). Red bars =1 mm. ★ original bone

3 and 6 months after operation with 3D-CT and histologic examinations of the operative regions. Bone regeneration was observed in groups 2-II and 2-III, with more extensive formation in group 2-III than in group 2-II (Fig. 13.2c–f). In group 2-I, no bone regeneration was observed (Fig. 13.2a, b). Moreover, in group 2-III at 6 months, new bone was observed connected to the existing cranial bone (Fig. 13.2f).

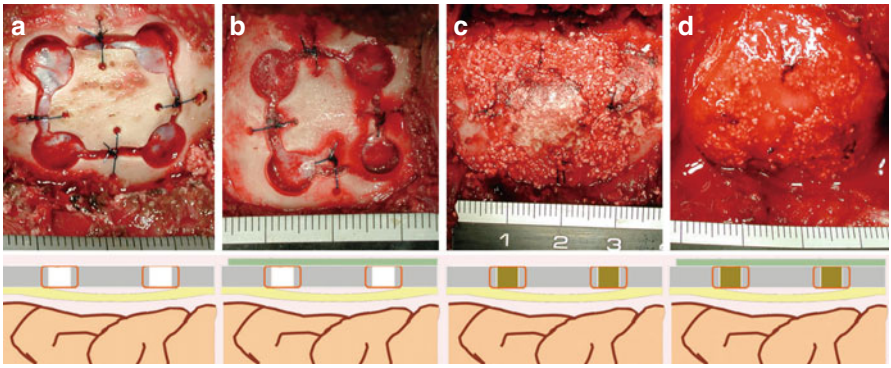

Fig. 13.3 Design of the operation, and intraoperative photographs. Group 3-I (**a**), group 3-II (**b**), group 3-III (**c**), and group 3-IV (**d**)

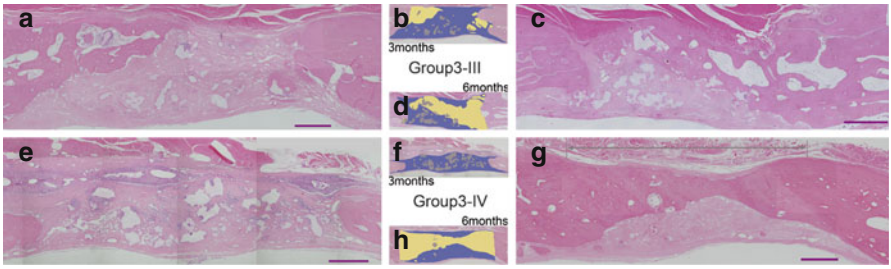

Fig. 13.4 Histology of group 3-III (**a**–**d**), and group 3-IV (**e**–**h**). Three-month model (**a**, **b**, **e**, **f**). Six-month model (**c**, **d**, **g**, **h**). The findings for the drilled hole for each model (**a**, **c**, **e**, **g**). Red bars =1 mm. Corresponding to figures, respectively (**b**, **d**, **f**, **h**). *Yellow areas* new bone, *gray areas* b-TCP, *blue areas* fibrous tissue, *star* original bone

This study showed that BSCs have the potential to promote cranial bone regeneration and confirmed the efficacy of a composite scaffold made of b-TCP combined with autologous bone fragments with fibrin glue.

13.4.3.3 Third Experiment [28]

Four experimental models were tested with or without BSM plus BSCs or CA membrane. In group 3-I, the original free bone flap was replaced in the defect (Fig. 13.3a). In group 3-II, after replacing the bone flap, the defect was covered with CA membrane (Fig. 13.3b). In group 3-III, BSM plus BSCs were used as a gap filler (Fig. 13.3c). In group 3-IV, BSM plus BSCs and CA membrane were applied (Fig. 13.3d). Histological examinations were performed 3 and 6 months after the operation. In groups I and II, no bone regeneration was observed but fibrous tissue intrusion was prevented in group II. More bone neogenesis was observed in group III than in group IV at 3 months ($P<0.05$) (Fig. 13.4a–d). At 6 months, the

regenerated areas were larger than those observed at 3 months, but the differences between groups III and IV were not statistically significant (Fig. 13.4e–g). This experiment showed that, as expected, CA membrane prevents excessive fibrous tissue intrusion and/or dislocation of a bone scaffold. However, use of a CA membrane did not always accelerate cranial bone regeneration. In addition, we found that there were other disadvantages associated with the use of a barrier membrane in cranial bone regeneration.

Accordingly, even though the barrier membrane acted as intended, levels of important bone regeneration regulatory factors within the barrier might be inadequate. In other words, the supplementary supply of vessels or regulatory factors from the superficial structures may be indispensable in cranial bone regeneration. If other GBR membranes are developed for cranial bone regeneration, these should be chosen to have the ability both to block fibrous tissue intrusion from the superficial layer and to be permeable to regulatory factors and vascularization.

13.4.4 Future Prospects

Collagen-coated b-TCP is a suitable scaffold material for cranial bone regeneration, as its structure needs to be maintained in position until bone regeneration is completed. To improve cranial bone regeneration, it is necessary to prevent fibrous tissue intrusion, while the supplementary supply of vessels or regulatory factors from the superficial structures needs to be maintained. Therefore, a new type of barrier membrane that can block fibrous tissue intrusion without blocking the supply of vessels or regulatory factors should be developed, or other approaches will need to be explored. Further, providing a generous amount of scaffold may compensate for the volume loss of regenerated bone. Changing the shape of the scaffold from a granule type to a block type may solve this problem, but a new problem then arises because it is very difficult to coat collagen into deeply positioned interconnected pores. The scaffolds that can provide suitable environments for cells are indispensable to cell survival and good regeneration. Therefore, further investigation is required with regard to cranial bone regeneration, including better scaffolds and suitable environments.

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Chapter 14
Salivary Gland Development and Regeneration

Tsuyoshi Kojima

Abstract Salivary glands are required for oral health and general well-being. In patients suffering from irreversible salivary hypofunction secondary to therapeutic radiation exposure or Sjögren's syndrome, available therapies are limited to salivary gland substitutes or parasympathetic stimulants, both of which show limited efficacy. The pathophysiologic basis of radiation-induced damage involves a reduction in viable acinar cells required for physiologic function, as well as a deterioration in acinar cell morphology. Together, these alterations lead to irreversible salivary gland dysfunction. Therefore, it is imperative to prevent damage to these acinar cells and to recover lost function of those cells after exposure to radiation. This chapter provides a brief overview of the current strategies being implemented for the prevention of radiation-induced damage, current strategies for the regeneration of acinar cells, and concludes with a basic review of salivary gland anatomy and development.

Keywords Salivary gland • Hyposalivation • Radiation • Sjögren's syndrome

14.1 Introduction

The parotid, submandibular, and sublingual glands represent three major pairs of salivary glands in the oral cavity that secrete 90 % of the total saliva produced. Saliva has many important roles. First and foremost, it aids in the lubrication of the oral cavity, allowing for the easy passage of food. In addition, it provides enzymes required for digestion and controls the growth of bacteria and fungi. As a result, it provides much of the innate host defenses required for the upper gastrointestinal tract, and its absence leads to numerous difficulties resulting from dry mouth conditions. These include halitosis, viral or bacterial throat infections, inflamed tongue,

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gingivitis, and aguesia, in addition to a significant impairment in quality of life [1–3]. Without question, the function of salivary glands is indispensable for oral and general well-being.

The production of saliva begins in the terminal branches of the salivary glands and is initiated by the acinar cells. These cells secrete water, electrolytes, and proteins into a duct that opens to the oral cavity. Other cells present in the area, including the ductal cells, reabsorb and regulate the contents of saliva. Three of the main clinical causes implicated in salivary hypofunction include exposure to therapeutic radiation in the head and neck area, Sjögren's syndrome, and iatrogenic side effects, of which radiation and Sjögren's syndrome lead to irreversible changes in acinar cells [4]. A treatment regimen for such irreversible salivary gland damage has not been designed, with research focused primarily on preventing acinar cell damage and regenerating these cells.

Of the literature focused on the clinical effects of radiation therapy, identifying ways to preserve the acinar cells is a priority. Post-therapeutic radiation for the treatment of head and neck cancer is administered to approximately 40,000 new patients each year in the United States and negatively affects more than 500,000 patients worldwide [5]. Radiation-induced hypofunction studies have evaluated the utility of intensity-modulated radiation (IMRT) to prevent intractable hyposalivation [6, 7]. Despite the fact that this therapy results in significant reduction in the incidence of xerostomia, about 40 % of these patients will go on to develop hyposalivation and consequential lifelong complaints [8, 9]. Furthermore, IMRT has yet to become accessible to all patients. Salivary gland transfer, an attractive method to prevent radiation-induced xerostomia, appears to be highly effective. Yet, only a select population of patients can receive this therapy. This population includes patients with primary tumors in the posterior oral cavity, oropharynx, nasopharynx, larynx, and hypopharynx, given that they lack lymphatic drainage into the Level 1 region of the neck [10–12]. Thus, there continues to be a need for further research to prevent salivary gland dysfunction after therapeutic radiation.

The evaluation of patients for radiation-induced hyposalivation following therapeutic radiation exposure is important. Approximately 12 million patients suffer from radiation-induced hyposalivation in the United States alone [5]. Unfortunately, the only therapies available are limited to salivary gland substitutes and parasympathetic stimulants, which show limited efficacies [13]. From a pathophysiologic standpoint, it appears that this irreversible dysfunction is attributed to a reduction in number and viability of acinar cells, in addition to morphological deteriorations instigated by inflammatory infiltration and fibrosis [14]. Thus, the recovery of acinar cells and the restoration/improvement of general tissue morphology are critical to treat severe intractable salivary gland dysfunction. Additional candidates for regenerating complex tissue structures and function include gene therapy, tissue engineering, and cell therapy. This chapter will provide a brief overview of current strategies being implemented for the prevention of radiation-induced damage, current strategies for the regeneration of acinar cells, and a review of salivary gland anatomy and development.

14.2 Anatomy

The parotid glands are wedged-shaped organs located inferior and anterior to the ears. They are the largest salivary glands found in the human body and drain to the buccal mucosa. The submandibular gland is only half the size of the parotid gland. It drains inferior to the tongue, while the sublingual gland drains through numerous ducts located in the floor of the mouth. Both the submandibular and sublingual glands lie in the floor of the mouth, inferior to the mucous membrane. Supplementary salivary glands, minor in comparison to the three described above, are scattered throughout the oral cavity in the buccal mucosa, lips, palate, and tongue. Each salivary gland is a branching ductal system that terminates into saliva producing acini. Acini secrete the majority of the fluids, ions, and proteins that constitute saliva. Furthermore, these acini are classified as either serous or mucosal, which are defined by the consistency of their secretions. For example, mucous acinar cells secrete mucins, which are negatively charged glycoproteins attached to carbohydrate chains. The negative ionic charge of mucins is what causes saliva to become viscous in nature. Serous acinar cells, on the other hand, lack mucin. They secrete numerous proteins, including amylase. Aside from these two major categories of acinar cells, some acini are classified as seromucous because they secrete both mucin and serous products. From an anatomical standpoint, acini will drain into intercalated ducts that will then drain into striated ducts. Myoepithelial cells that surround the acini will contract to expel saliva from the acini and into the ductal system. Myoepithelial cells are stimulated by both parasympathetic and sympathetic nervous innervation. As the saliva begins to move through the ductal system, additional proteins are secreted and the ion composition changes accordingly. Of the different types of salivary glands in the body, the parotid glands are composed exclusively of amylase-rich serous cells, while the submandibular and sublingual glands contain both serous and mucous acini. The minor salivary glands are composed mainly of mucous acini [1, 2, 15, 16].

14.3 Development

The process of salivary gland organogenesis represents a type of branching morphogenesis. The first visible change to be noticed throughout this period is the initiation of a thickened oral epithelium. This occurs in the prebud stage during embryonic weeks 6–8. This thickened epithelium protrudes into the mesenchyme to form an initial bud, which then solidifies with concurrent epithelial proliferation. Subsequently, the primordial salivary gland continues to produce epithelial clefts and branches that then form into bush-like clusters of buds during the pseudoglandular stage. Each epithelial cleft forms 4–5 buds and continues branching to produce a multi-lobed organ. During the canalicular stage, the early lobes and ductal system arise via apoptosis of epithelial cells. Specifically, the apoptosis occurs

within the branches and terminal buds to form primal acini and myoepithelial-associated distal ducts. By the 7th embryonic month, the terminal bud stage begins as the acini reach maturation. The presence of intercalated ducts is apparent as well [1, 2, 15–17].

The salivary gland continues to differentiate after birth, and differentiates into its terminal granular convoluted tubules at puberty [1]. Interestingly, the salivary gland epithelial cells never cease to proliferate throughout each stage of organogenesis, including the terminal bud stage. This occurs in order to limit the number of excretory and intercalated ducts [17, 18]. The establishment of nerve endings and the blood supply occurs throughout the entire process of branching morphogenesis and with gland cellular differentiation.

Our knowledge of salivary gland development stems from work conducted on the submandibular gland in the mouse model. This gland has been shown to undergo a branching morphogenesis consistent across other major salivary organs. The literature reports a functional integration of growth factors, cytokines, and transcriptional growth factors specific to each developmental stage. Furthermore, the cooperation of complex organs such as the pancreas and lung is required for signaling pathways to take place, leading to appropriate cellular proliferation, quiescence, apoptosis, and differentiation [17].

14.4 Prevention

When patients are treated with radiation therapy for head and neck cancer, their salivary glands are at risk of permanent damage. Radiation therapy results in the generation of free radicals in an attempt to halt progression of tumorigenesis. Radiation is thought to damage cellular DNA, and, through repeated cycles of cellular division, leads to the accumulation of cellular damage that kills the tumor cells or reduces their proliferation. Since the salivary glands are also located in the head and neck region, they also suffer the damaging effects of radiation. With the slower turnover rate of salivary glands compared to tumor cells, specifically of at least 60 days, their function is severely compromised. Thus, it is not the DNA damage to progenitor cells or stem cells that is responsible for the acute dysfunction of the salivary glands, but rather the radiation itself [19, 20]. Given that apoptosis of acinar cells occurs in the late phase of radiation treatment [21], it may be possible to restore salivary gland function should acinar cells be able to regenerate during this time period.

Unfortunately, no ideal radioprotective treatment has been developed. There are numerous studies that have attempted to identify molecular factors that may dampen radiation-induced damage to the salivary glands. Our research group has evaluated the protective effects of basic fibroblast growth factor (bFGF) toward radiation damage in the salivary gland [22]. bFGF is a well-known mitogen that accelerates tissue regeneration through its ability to induce angiogenesis, granulation tissue formation, and epithelial cell proliferation [23, 24]. We found that the administration of bFGF improved hyposalivation at 8 weeks after irradiation (Fig. 14.1). Histologic

analysis confirmed that bFGF-treated glands contained more acinar cells compared to untreated glands and that the apoptotic response to irradiation, examined at 1–2 days following irradiation, was reduced (Fig. 14.2). Thus, the inhibition of radiation-induced apoptosis may serve as a protective mechanism against the death of progenitor cells or stem cells and demonstrates the potential to promote the proliferation of acinar cells and improve salivary function (Fig. 14.3). Moreover, the pro-paracrine effect of HGF and VEGF may also play an important role in salivary gland regeneration [25, 26] (Fig. 14.4).

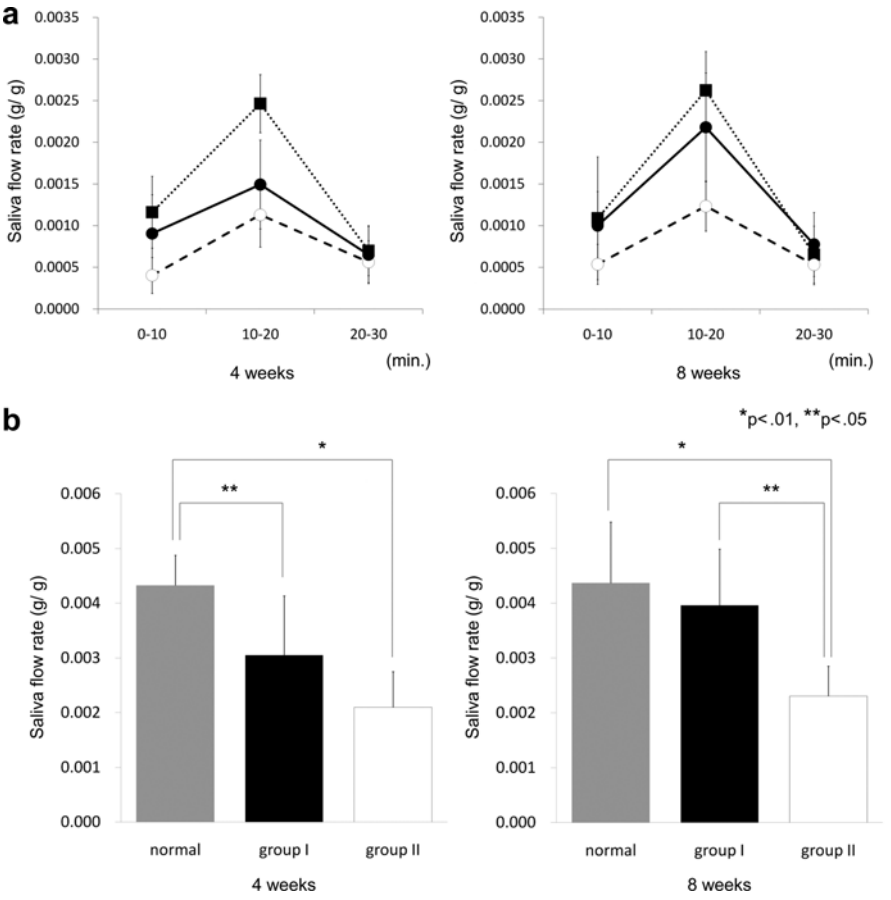

Fig. 14.1 Saliva flow rate at 4 and 8 weeks after irradiation. (**a**) The saliva flow rate was measured in 10-min intervals for 30 min after pilocarpine injection. (**b**) Saliva flow rate for 30 min after pilocarpine injection. The normal, nonirradiated, and untreated group is indicated by the *black squares*. Group I was treated with basic fibroblast growth factor after irradiation, which is indicated by *black circles*. Group II was not treated after irradiation, which is indicated by *white circles*. In the 4-week comparison: normal (13 weeks old, $n=8$), Group I ($n=8$), Group II ($n=8$). In the 8 week comparison: normal (17 weeks old, $n=8$), group I ($n=8$), group II ($n=8$) (Image from Published Paper [22])

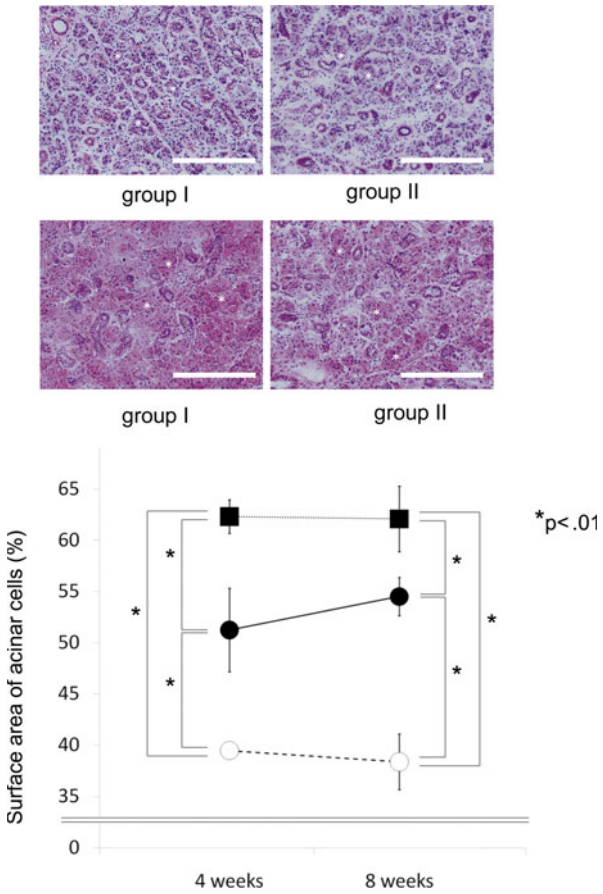

Fig. 14.2 Histologic findings. (**a**) Hematoxylin and eosin staining at 4 weeks after irradiation. (**b**) Periodic acid-Schiff staining at 4 weeks after irradiation. Scale bar: 200 lm. *Asterisk* indicates acinic cells. (**c**) The percentage of the surface area comprised of acinar cells was calculated from four mice in each group. *Black squares* indicated the normal, nonirradiated, and untreated group. *Black circles* indicate Group I. These animals were treated with basic fibroblast growth factor after irradiation. *White circles* indicate Group II. These animals were untreated after irradiation (Image from Published Paper [22])

Other studies have shown similar results using molecular factors. Maedina et al. reported a complete reversal in radiation-induced hyposalivation through the use of histamine [27]. They demonstrated that appearance and structure was preserved, and speculated that histamine may suppress apoptosis and prevent suppression of cellular proliferation [27]. Another protein that shows radioprotective properties is heat shock protein 25. Lee et al. demonstrated its ability to inhibit radiation-induced apoptosis and mediate radiation-induced fluid loss in the salivary gland although radiation inhibits salivary gland aquaporin 5 expression [28]. Similarly, Zheng et al. demonstrated the prevention of salivary hypofunction with the use of a keratinocyte growth factor (KGF) gene transfer model. KGF promotes an increase in endothelial cell

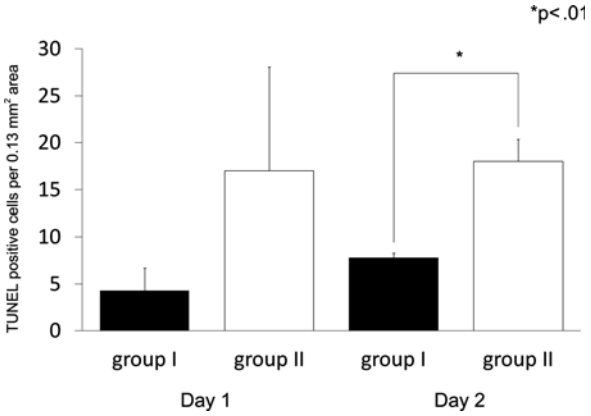

Fig. 14.3 Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL)–positive cells. TUNEL-positive cells were counted in five randomly chosen fields from each mouse. The number of scored cells was averaged for each mouse. Five samples were counted for each group and day. Group I were treated with basic fibroblast growth factor after irradiation. Group II were untreated after irradiation (Image from Published Paper [22])

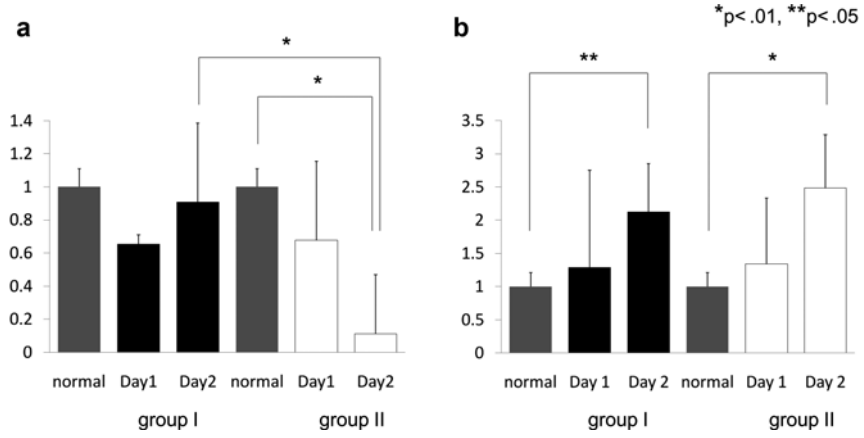

Fig. 14.4 Expression of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) mRNA. Quantitative real-time polymerase chain reaction was performed in a mixture of cDNA samples, TaqMan Universal PCR Master Mix, and the TaqMan probes and primers for HGF (**a**) or VEGF (**b**). The *gray bar* indicates expression in the normal, nonirradiated, and untreated group. Group I (*black bar*) was treated with basic fibroblast growth factor after irradiation. Group II (*white bar*) was untreated after irradiation. Day 1 indicates 1 day after irradiation, and Day 2 indicates 2 days after irradiation. Four samples were assayed from each group and day (Image from Published Paper [22])

number, augments cellular proliferation, and increases the number of salivary gland progenitor stem cells [29, 30]. Additional research efforts in preventing acute radiation-induced damage include hyperbaric oxygen therapy [31], cytokine treatment [32], and injection of the heparan sulfate mimetic regenerating agent OTR4120 [33].

The above studies show the importance of protecting salivary gland progenitor stem cells from radiation-induced free radicals. Although the cytoprotectant, amifostine, is approved by the Food and Drug Administration with proved efficacy for cancer treatment, it does not constitute the standard treatment due to severe side effects [34]. Given that a priority is placed on treating cancer rather than side effects associated with radiation, it is challenging to find effective, side effect-free regimens. However, priority in devising this type of regimen is essential, unless salivary gland cells can be regenerated. Finding a solution to radiation-induced damage is important.

14.5 Regeneration

Because it is very difficult to reverse salivary gland dysfunction due to radiation or Sjögren's syndrome, several strategies have been proposed to mediate its effects. These include gene therapy, tissue engineering, and cell therapy, all of which are considered excellent models for regenerating complex tissues structures and restoring function. From a gene therapy approach, Baum et al. developed a novel way to use aquaporin-1 cDNA as a treatment strategy in current patients with radiation-induced salivary dysfunction [35, 36]. He devised a recombinant adenoviral vector to mediate the gene transfer and administered it to patients through a phase I/ II clinical trial. From a tissue-engineering standpoint, a three-dimensional (3D) culture system using hyaluronic acid (HA) hydrogels was created to artificially simulate the salivary gland. The seeded cells produced lobular-like acini that stained positive for alpha-amylase [37, 38]. Furthermore, poly-lactic-co-glycolic acid (PLGA) has been utilized as a scaffold to promote the self-organization of cells into a 3D construct [39–41]. Ogawa et al. has demonstrated the successful replacement of salivary gland function through the transplantation of a bioengineered salivary gland germ [42]. These innovative strategies demonstrate the potential of inducing salivary gland regeneration through tissue engineering techniques. The utilization of stem cell therapy, however, appears to hold the greatest promise. Stem cell therapy has already demonstrated successful gain of function in irradiated salivary glands. Stem cells, characterized by their ability to yield substantial cellular proliferation and the differentiation into various cell types, can be harvested from tissue-specific sources or non-tissue-specific sources. The latter represents a more useful way to collect the number of cells required to demonstrate an efficacious result. Lombaert et al. has shown that bone marrow-derived stem cells (BMSCs) ameliorate acinar cell loss and vascular damage [43]. Sumita et al. demonstrated that BMSCs, once transplanted into the mouse-tail vein, also led to the regain of function in irradiated salivary glands [44, 45]. Our research group reported previously that adipose tissue-derived stem cells (ADSCs) can ameliorate radiation-induced salivary dysfunction [46]. ADSCs represent an additional source of stem cells given the feasibility in collecting large amounts of these cells through the use of minimally invasive

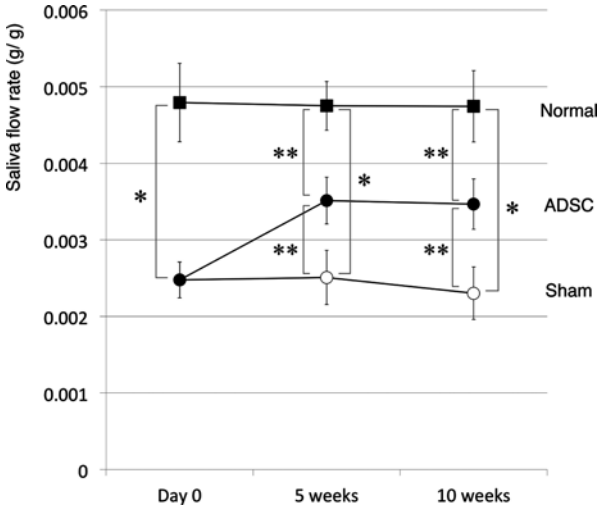

Fig. 14.5 Saliva flow rate at days 0, 5, and 10 weeks after irradiation. The saliva flow rate was assessed for 30 min after pilocarpine injection. Normal group (*black square*); ADSC group (*black circle*): sham ADSC group (*white circle*). *$P < .01$, **$P < .05$, one-way factorial ANOVA, post hoc Fisher PLSD (Image from Published Paper [46])

procedures compared to the harvesting of BMCs [47]. Use of ADSCs in our study led to an improvement in salivary flow rate compared to the sham group (Fig. 14.5). Furthermore, the proliferation of blood vessels (Figs. 14.6 and 14.7), paracrine effects (Fig. 14.8), and the differentiation of ADSCs into blood endothelial cells and ductal cells were observed (Fig. 14.9). Use of ADSCs appears to result in angiogenesis, improved production of mucin and amylase, decrease in number of atrophied acinar cells, reduction in fibrotic scarring, and improved blood flow. More recently, Lim et al. demonstrated the differentiation of infused ADSCs into salivary gland cells, highlighting the remarkable capability that stem cell research holds in treating radiation-induced salivary dysfunction [47].

The use of undifferentiated stem cells from the salivary gland itself has been shown to repair the function of salivary glands damaged by radiation [9, 48–52]. Radiation diminishes the capability of these stem cells to differentiate into the required cell lines, suggesting that the main reason for irreversible hyposalivation is inadequate production of acini cell precursors. Although tissue cultures are required to expand the number of undifferentiated stem cells sufficient to restore salivary function, they showcase a remarkable capability in regeneration with the provision that the required quantity of cells can be harvested. Thus, it is our hope that this innovative method constitutes the basis of treatment for patients presenting with xerostomia in the future.

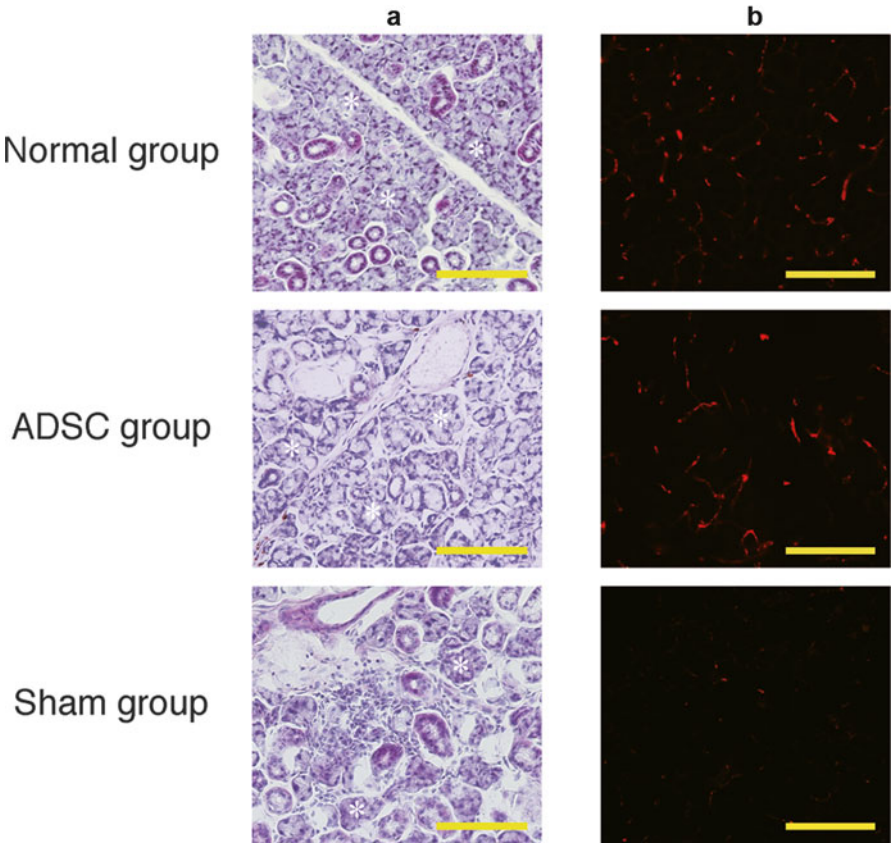

Fig. 14.6 Improvement in salivary gland tissue morphology and angiogenesis. H&E staining (**a**) and immunohistochemistry for CD31 (**b**, *red signal*) is shown at 10 weeks after administration/transplantation. Scale bar: 100 μm; *Acinic cells (Image from Published Paper [46])

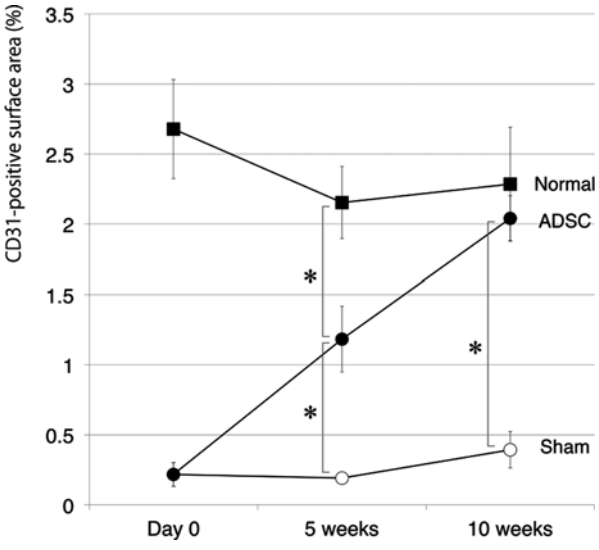

Fig. 14.7 Percentage of CD31-positive surface area in the salivary glands. The percentage of the total surface area positive for CD31 immunoreactivity was measured and calculated in 10 randomly chosen fields from sectioned salivary glands from each mouse. The percentage of the scored area was averaged for each mouse. *$P < .01$, one-way factorial ANOVA, post hoc Fisher PLSD (Image from Published Paper [46])

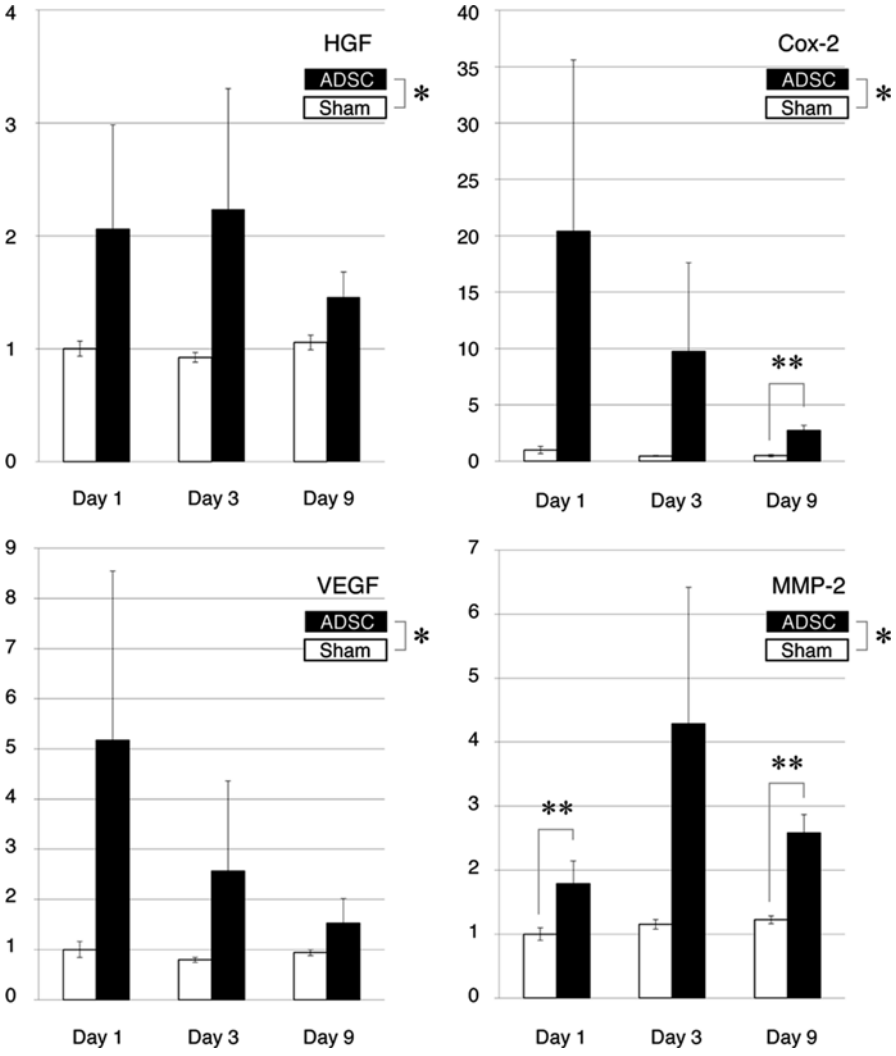

Fig. 14.8 Paracrine effects of ADSCs. Quantitative RT-PCR was performed in a mixture of cDNA samples, TaqMan® Universal PCR Master Mix, and the TaqMan® probes and primers for HGF, VEGF, Cox-2, and MMP2. Black bar (*ADSC*): ADSC group; white bar (Sham): sham group. *Day 1* indicates 1 day after the administration of saline or ADSC cells; *Day 3* indicates 3 days after administration; *Day 9* indicates 9 days after administration. Five samples were assayed from each group and day. *$P < .01$, two-way factorial ANOVA, post hoc Fisher PLSD. **$P < .01$, Student's *t*-test (Image from Published Paper [46])

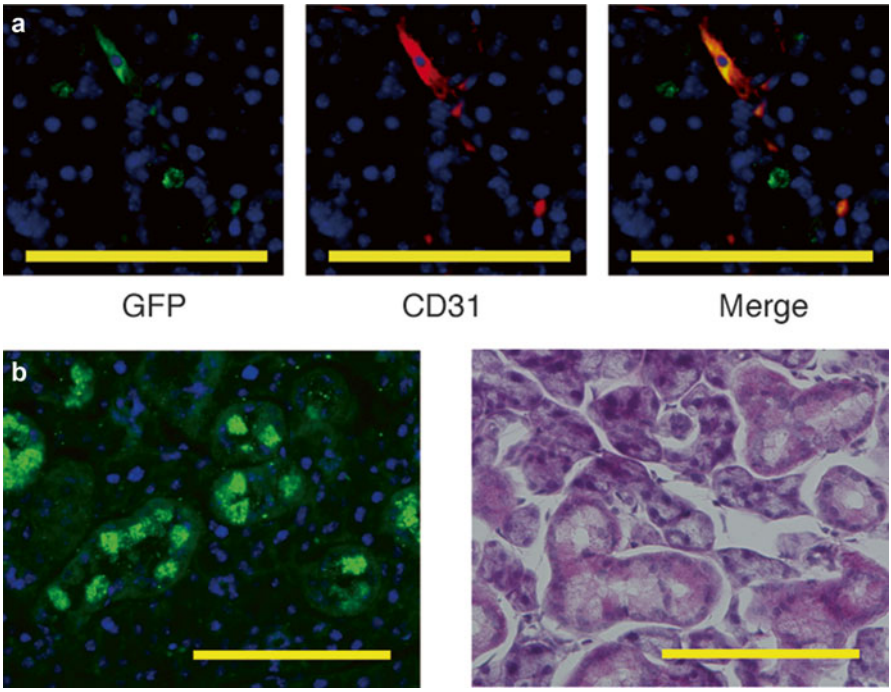

Fig. 14.9 Differentiation of transplanted ADSCs into blood endothelial cells and ductal cells. (**a**) Immunohistochemistry for GFP expression was used to identify transplanted ADSCs, and CD31 expression was used to identify blood endothelial cells. The merged image shows double positive cells. (**b**) Transplanted ADSCs are positive for GFP expression (*green signals*). Scale bar: 100 μm (Image from Published Paper [46])

14.6 Conclusion

Irreversible salivary gland hypofunction occurs from therapeutic radiation exposure to the head and neck area or Sjögren's syndrome, both of which will lead to irreversible changes in salivary gland acinar cells.

In patients requiring radiation therapy, it is difficult to administer therapeutic, radioprotective regimens given their reduced efficacy in treating the cancer. Therefore, research efforts have centered on utilizing molecular factors, gene therapy, tissue engineering, and/or cell therapy to protect acinar cells from harmful radiation effects or initiate a regenerative process that will allow the salivary gland to function properly after either radiation or autoimmune conditions. Through the use of these innovative techniques, it is our hope that they will constitute the standard treatment of care for patients that present with salivary gland hypofunction regardless of the cause and prevent the sequelae of negative events that continue to remain a significant public health concern.

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Chapter 15
Regeneration of the Trachea

Shin-ichi Kanemaru, Koich Omori, Masaru Yamashita, and Tatsuo Nakamura

Abstract We previously conducted a study in which a tracheal prosthesis was placed in dogs. The tracheal prosthesis was made from tissue regenerated from autologous cells. We successfully regenerated the mucosal epithelium and cilia along the tracheal lumen. The maximum length of the defect was six tracheal rings. Based on the results of this study, we conducted studies on clinical application of such prosthesis in human subjects with tracheal stenosis or defect after cancer surgery or injury. These studies have yielded good results. However, in clinical application for pediatric patients, a regenerated trachea must grow as the patients grow. Thus, it is necessary to develop a scaffold that has the strength to maintain a patent tracheal lumen, enables cartilage regeneration to progress, and completely degrades when regeneration is complete. There is much anticipation in future studies using cultured cartilage tissue and iPS cells.

Keywords Regeneration of trachea • Tracheal prosthesis • Tissue engineering • Tracheal defect • Stenosis of trachea

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15.1 What Is Regenerative Medicine?

Regenerative medicine can be defined as a branch of medical science that studies the regeneration of irreversibly damaged tissues and organs using cells (stem cells). The objective of regenerative medicine is to regenerate morphologically and functionally normal tissue.

There are three elements of tissue engineering that are essential for tissue regeneration (Fig. 15.1): cells, scaffold, and regulatory factors. Cells form the foundation of tissue regeneration, a scaffold is needed for the growth of cells, and regulatory factors control their growth. Regeneration can proceed when these elements are in an appropriate regenerative environment. Research in regenerative medicine investigates how to prepare and select these elements and how to produce a favorable regenerative environment.

In the original method of tissue engineering, these three elements were placed in a Petri dish to prepare the target tissue and the resulting tissue was implanted into a living body [1]. Absolute requirements for successfully engineered tissue are the establishment and maintenance of a blood supply to the implanted tissue and the survival of the tissue. However, if the tissue is larger than a certain size, a good blood supply is difficult to maintain in the implanted tissue.

A concept called "in situ tissue engineering" (in vivo regeneration) was developed by the Institute for Frontier Medical Sciences at Kyoto University. This concept involved direct placement of these three elements into the living body for regeneration. According to in situ tissue engineering, if cells can be supplied by

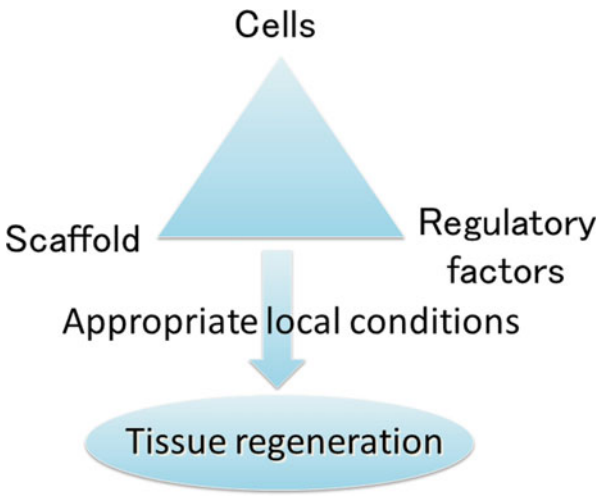

Fig. 15.1 Three elements of tissue engineering. Cells: stem cells (such as ES cells and mesenchymal stem cells) and progenitor cells. Scaffold: natural polymers such as collagen used as artificial extracellular matrix, synthetic polymers such as polyglycolic acid, and inorganic compounds such as hydroxyapatite. Regulatory factors: hepatocyte growth factor (HGF), basic fibroblast growth factor (b-FGF), nerve growth factor (NGF), various interleukins, and interferons

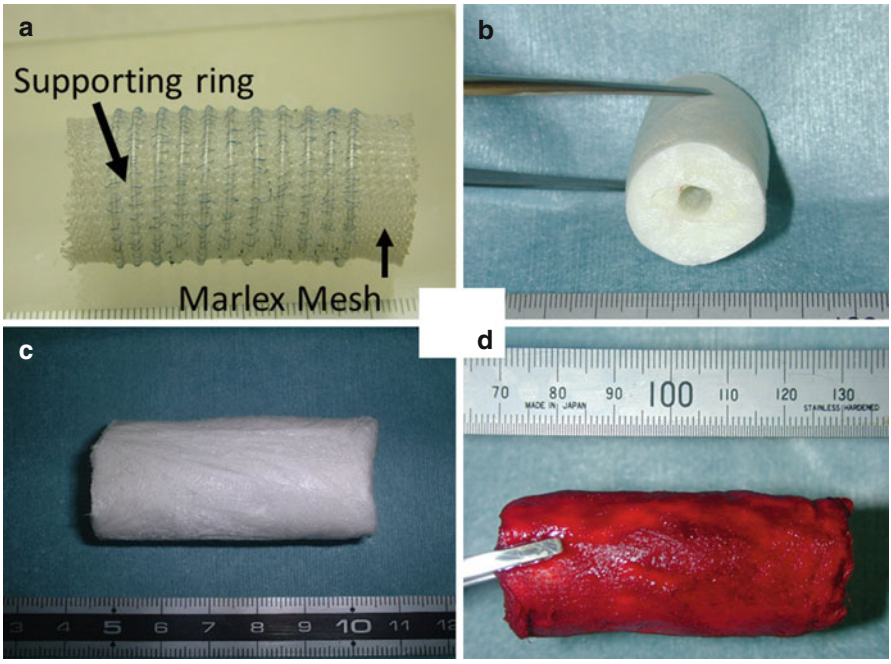

Fig. 15.2 Tracheal prosthesis. (**a**) Framework of tracheal prosthesis. (**b**, **c**) Tracheal prosthesis was composed of a polypropylene frame and a collagen sponge scaffold. The prosthesis can be sterilized as is and stored. (**d**) The prosthesis was soaked in blood. As a result, the collagen sponge became compressed and the lumen enlarged, resulting in an airtight condition

the surrounding tissue, only addition of scaffold and regulatory factors is needed for cells and blood vessels to migrate from the surrounding tissue into the scaffold. Subsequently, tissue regeneration proceeds as the cells grow in the shape of the scaffold. This method is currently used for clinical application of tracheal regeneration.

15.2 Development of Tracheal Prosthesis

In the 1970s, Professor Grillo showed that tracheal reconstruction can be performed with end-to-end anastomosis if the tracheal defect was 6 cm or less [2]. However, this surgical procedure places a large burden on patients, and the patients must maintain their necks in a flexed position for approximately 1 month after surgery. Various tracheal prostheses were developed to enable tracheal reconstruction without a significant burden on patients with cervical flexion, but many of these efforts were unsuccessful.

In Japan, research on tracheal prosthesis began to progress in 1980. Tracheal prostheses currently in clinical application are prostheses developed using tissue regenerated from autologous cells and subsequently further improved. Figure 15.2 shows the construction of such prosthesis. Its supporting structure is made from a

fine Marlex mesh cylinder (BARD mesh), which is reinforced with polypropylene monofilament spiral. The cylinder is then covered with a sponge made of atelocollagen extracted from porcine skin. This atelocollagen sponge has a thin film, multilocular microstructure.

The trachea has a hollow interior and is supported by cartilage in its walls. The trachea can maintain its shape because cartilage rings give resistance to external compression and negative pressure caused by coughing. Therefore, tracheal regeneration requires scaffold materials with certain strength. Marlex mesh alone does not provide sufficient strength to maintain a patent tracheal lumen. Therefore, polypropylene was used for reinforcement. In our studies, Marlex mesh and polypropylene reinforcement are fusion-bonded by heat and secured together using 7-0 surgical sutures at a 2 mm interval.

Shimizu and Nakamura et al. developed a tracheal prosthesis whose polypropylene monofilament surface was molecularly modified to increase its tissue compatibility. Specifically, they used plasma surface treatment on the prosthesis to create chemically reactive groups to which collagen molecules could covalently bond [3].

The tracheal prosthesis was soaked in blood. Subsequently the remaining tracheal stumps were inserted into the prosthesis and secured using an absorbable suture (Fig. 15.3). In the implanted tracheal prosthesis, fibroblasts migrate from the surrounding tissue into the atelocollagen scaffold. Fibroblasts secrete collagen which replaces atelocollagen, and regeneration of autologous tissue occurs. Regeneration is complete within 1–6 months depending on the length of the defect. Experiments using beagles have shown that tracheal mucosal epithelium and cilia were regenerated along the lumen of the tracheal prosthesis (Figs. 15.3 and 15.4). Tracheas were resected and complete regeneration of the epithelium and cilia was observed even at the tracheal bifurcation (Fig. 15.4). The longest segment of trachea replaced by prosthesis was 9 tracheal rings in length [4, 5].

15.3 Clinical Application of the Tracheal Regeneration

A 5-year study on dogs has demonstrated the long-term safety of the tracheal prosthesis made using autologous tissue replacement [6]. Clinical application of this tracheal prosthesis began in 2002 after the review and approval by the ethics committees of the Department of Otolaryngology, Head and Neck Surgery, at Kyoto University Hospital, the Department of Otolaryngology at Fukushima Medical University Hospital, and the Department of Otolaryngology at Kitano Hospital in Osaka. This tracheal prosthesis is presently used for reconstruction of cervical tracheal defects due to tracheal invasion of thyroid cancer and for repair of tracheal stenosis due to various causes [7, 8]. Good results have been obtained from such procedures (Fig.15.4).

A tracheal prosthesis is soaked in blood before it is implanted into the area of the tracheal defect. The prosthesis becomes airtight, and migration of the surrounding cells is also facilitated by this process. In an animal experiment, tracheal prostheses were soaked with peripheral blood, bone marrow aspirate, or bone marrow-derived mesenchymal stem cells. In the prostheses with mesenchymal stem cells, there was

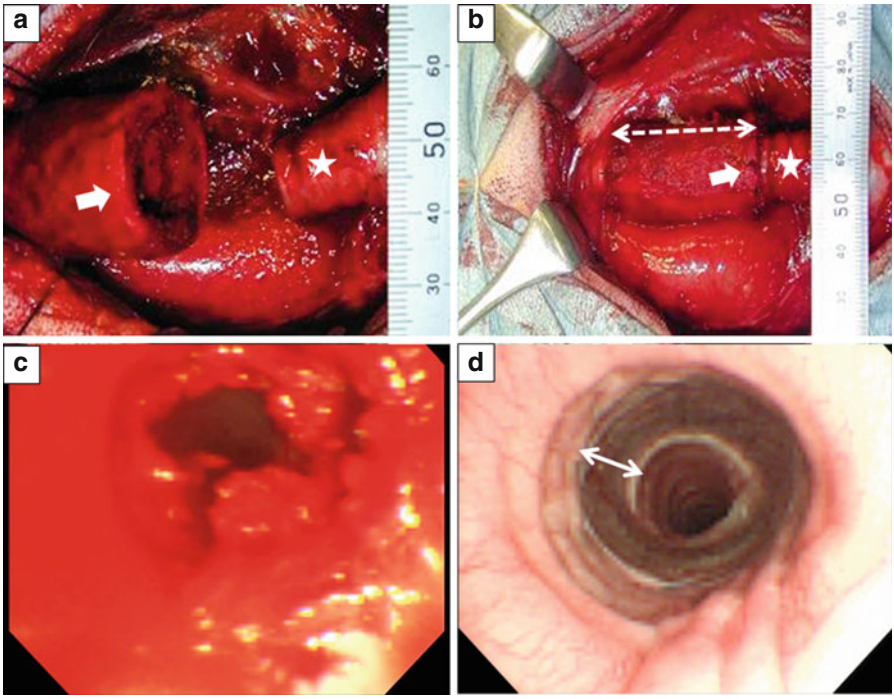

Fig. 15.3 Suture fixation of tracheal prosthesis (dog). *Star*: Caudal trachea, *solid single-headed arrow*: end of the tracheal prosthesis, *dotted double-headed arrow*: tracheal prosthesis, *solid double-headed arrow*: recipient site of tracheal prosthesis after regeneration. (**a**) Caudal tracheal prosthesis before suture fixation. (**b**) The trachea was inserted into the ends of the tracheal prosthesis, and suture fixation was performed. (**c**) Endoscopic findings of tracheal lumen soon after implantation of tracheal prosthesis. (**d**) Patency of the tracheal lumen was maintained and epithelialization was complete at 10 months after implantation

tracheal cartilage regeneration in some areas and differentiation into submucosal tissue [9, 10]. Other experiments are being conducted on regeneration using mesenchymal stem cell transplantation and iPS cells for future clinical application.

In clinical practice, a long, circumferential tracheal segment is very rarely replaced using a tracheal prosthesis. In our experience, the longest defect was 5 cm in length and three-fourths the circumference of the trachea, and the defect was caused by tracheoesophageal invasion by thyroid cancer (Fig. 15.5). When a tracheal prosthesis is used for a partial-circumference defect, the disadvantage is a slight loss of strength of the prosthesis. The advantage is earlier epithelialization of the lumen of the prosthesis because a portion of the normal trachea remains (Fig. 15.6) [7]. In addition, when a tracheal prosthesis is used in patients with tracheal invasion by cancer, there are the following advantages:

1. Tracheal resection at the site of tracheal invasion can be performed with a wide margin of safety.
2. Harvesting of autologous tissue is unnecessary for reconstruction of tracheal defect.

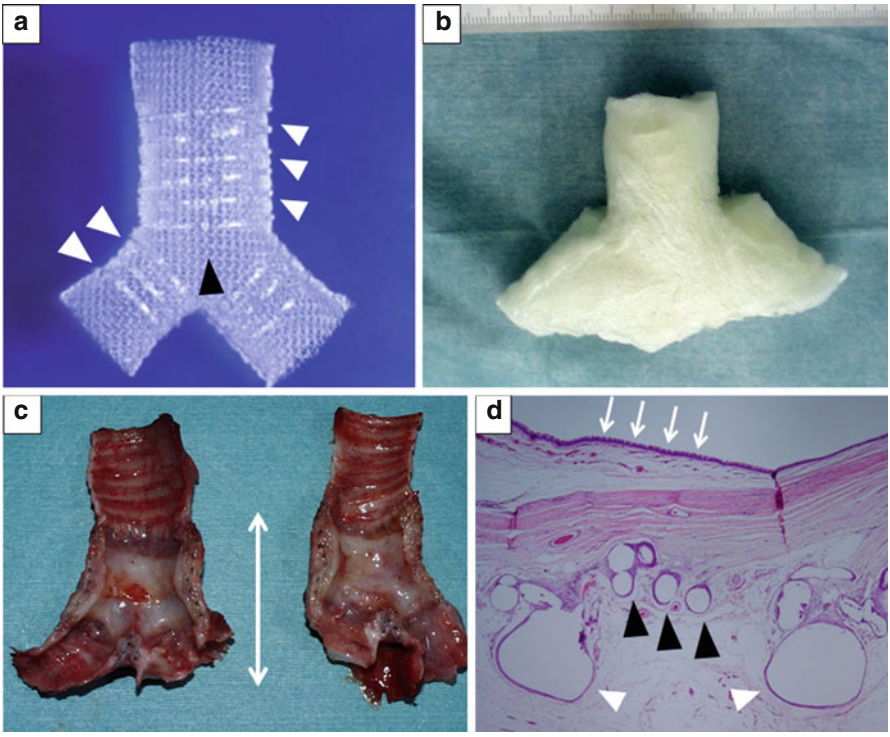

Fig. 15.4 Regeneration at the tracheal bifurcation (dog). *White arrowhead*: polypropylene reinforced ring, *black arrowhead*: Marlex mesh, *solid double-headed arrow*: recipient site of tracheal prosthesis after regeneration, *solid single-headed arrow*: tracheal epithelium and cilia in the area of regeneration. (**a**) Framework at the tracheal bifurcation of the prosthesis. (**b**) Tracheal bifurcation of the prosthesis composed of a polypropylene frame (**a**) and a collagen sponge scaffold. (**c**) Cross-section specimen showing the tracheal prosthesis lumen 1 year after implantation. (**d**) Tissue sample of recipient site of the tracheal prosthesis in another dog (HE staining)

3. Tracheotomy is unnecessary, surgery can be performed in one stage, and the length of the hospital stay is short.
4. Esthetically superior results can be obtained because there is minimal deformity in the cervical region.

Thus, the major advantages of tracheal prosthesis are that the possibility of recurrence of cancer is reduced and discovery of recurrence can be made easily [7].

15.4 Problems of the Tracheal Prosthesis

When a tracheal prosthesis is made using autologous tissue replacement, a problem arises from the use of polypropylene monofilament to maintain the strength of the trachea. Monofilament does not degrade when autologous tissue replacement

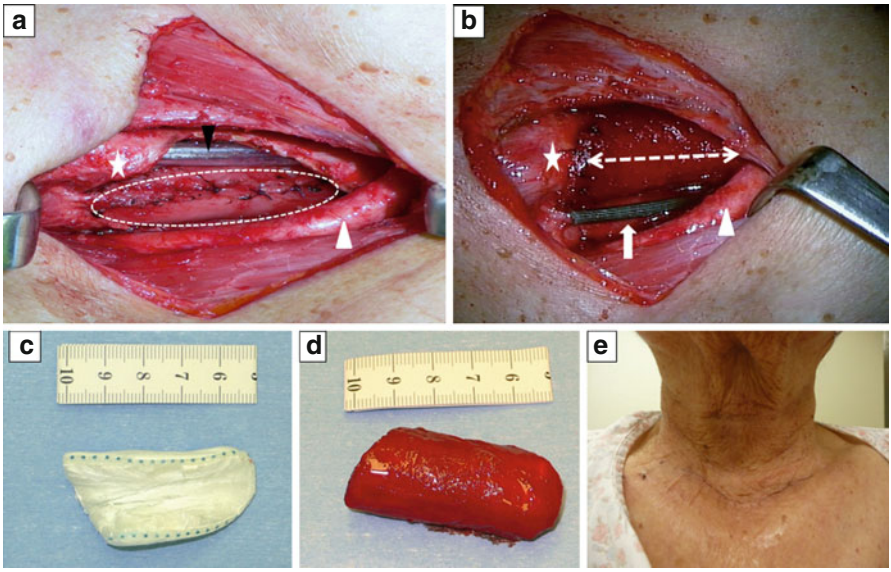

Fig. 15.5 A case of simultaneous regeneration of the trachea and recurrent laryngeal nerve (human: thyroid cancer T4NxMo). *Star*: cranial trachea, *Arrowhead*: common carotid artery, *dotted ellipse*: a portion of the esophagus that was resected (because of cancer invasion) and sutured, *dotted double-headed arrow*: implanted tracheal prosthesis, *single-headed arrow*: area of the recurrent laryngeal nerve (5 cm) reconstructed using an artificial nerve conduit. (**a**) Tracheal defect after resection of thyroid cancer (resection from the first to fifth tracheal ring). (**b**) After suture fixation of the tracheal prosthesis and artificial nerve conduit in the area of the tracheal defect and in the area of recurrent laryngeal nerve resection, respectively. (**c**) Luminal side of the tracheal prosthesis trimmed to match the tracheal defect. The cross sections of polypropylene reinforced rings are shown as dotted lines on the cut surface of the prosthesis. (**d**) Tracheal prosthesis soaked in blood. (**e**) Neck of a patient (78-year-old woman) 3 weeks after surgery. Tracheotomy was unnecessary and there was minimal deformity in the cervical region

progresses and remains unchanged in the living body. Thus, the lumen will not become larger than the size of the trachea established by the initial framework. Therefore, a major problem is that this prosthesis cannot be used in pediatric patients. The regenerated trachea will need to grow with the growth of patients if it is to be used in clinical application in pediatric patients. To solve this problem, it will be necessary to develop a scaffold that enables cartilage regeneration while maintaining the strength of the prosthesis to keep a patent tracheal lumen and that completely disappears after the completion of regeneration.

Macchiarini et al. used a method involving an allograft. A donor trachea was harvested from a cadaver and decellularized. The resulting tracheal cartilage structure was seeded with the patient's autologous chondrocytes and epithelial cells to prepare a cultured trachea. This trachea was implanted into the patient [11]. If this trachea can achieve long-term success, then it might be applicable in pediatric patients. Since only short-term results are currently available, we cannot determine its applicability in pediatric patients. In any case, major problems are cartilage regeneration and maintenance of strength [10, 12].

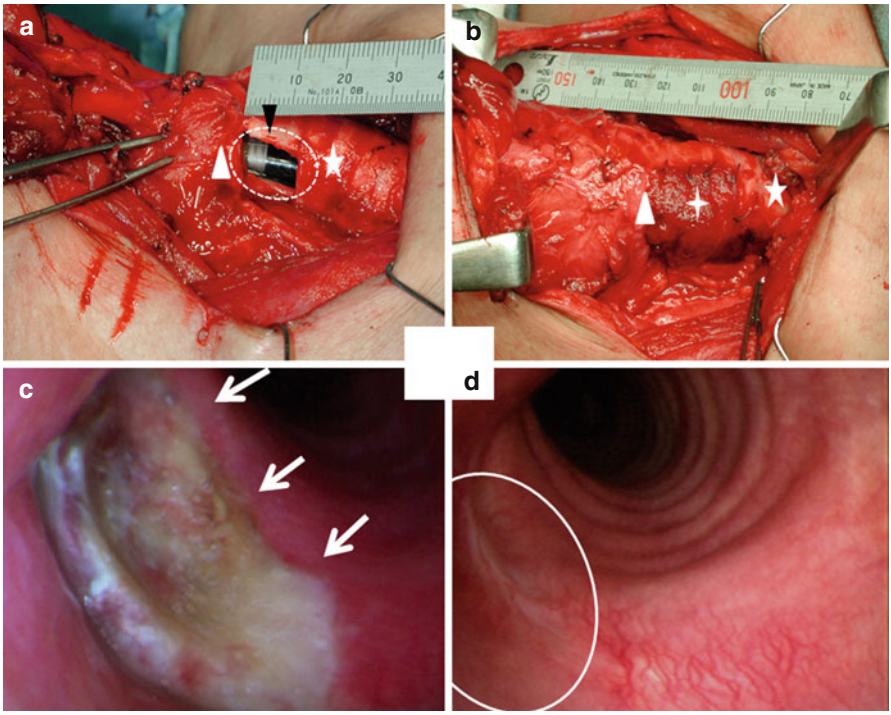

Fig. 15.6 A case of clinical application of tracheal prosthesis for partial-circumference defect (human thyroid cancer T4N1bMo). *Star*: caudal trachea, *white arrowhead*: cricoid cartilage, *black arrowhead*: intubation tube, *dotted ellipse*: area of the tracheal defect after resection because of cancer invasion, *white cross*: implanted tracheal prosthesis, *arrow*: luminal surface of the implanted tracheal prosthesis, *solid ellipse*: luminal surface of the implanted tracheal prosthesis. (**a**, **b**) Clinical case of tracheal invasion by thyroid cancer (71-year-old man). (**c**) Progression of epithelialization 1 week after surgery. (**d**) One year after surgery, the regenerated area was almost the same as the normal tissue

15.5 Regeneration of the Tracheal Cartilage

Animal studies have been conducted on tracheal prostheses made using autologous tissue replacement. Histological examination showed partial regeneration of cartilage [11, 12], but the regeneration was not around the entire tracheal circumference and a patent lumen was not maintained.

Many researchers, including Omori et al., have been conducting studies using cultured cartilage and iPS cells to regenerate tracheal cartilage [13, 14]. Nomoto M. et al. used chondrocytes cultured from autologous cartilage in a rabbit model [14]. These chondrocytes were seeded into a tracheal prosthesis and cultured. The resulting prosthesis was implanted into tracheal defects in rabbits. Fourteen weeks after implantation, the recipient sites showed regeneration of cartilage along almost the entire length of the defect (Fig. 15.7). Although there is much potential in cartilage regeneration (the ultimate objective of tracheal regeneration), cartilage regeneration has not yet reached the stage of wide clinical application.

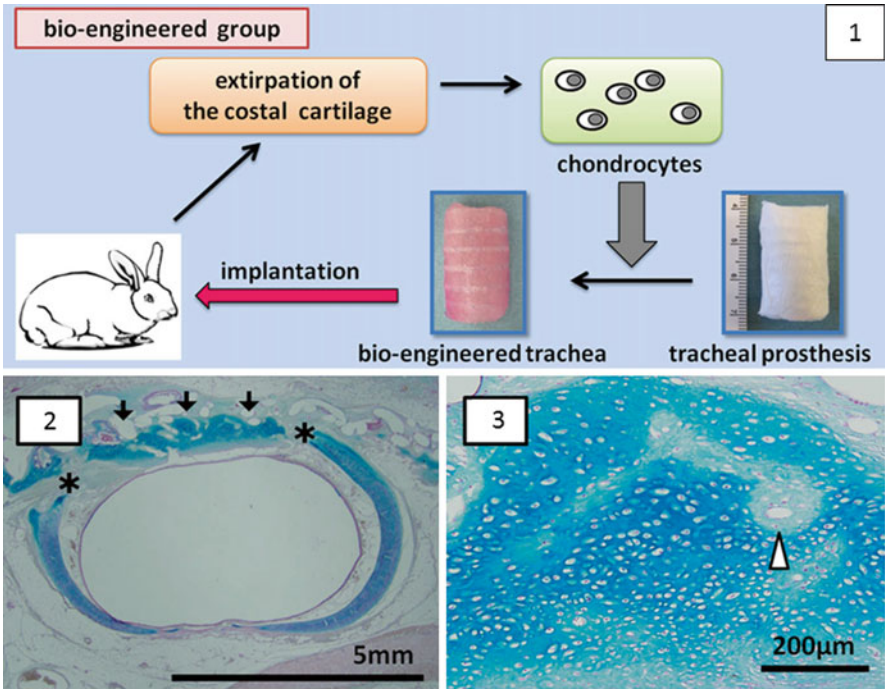

Fig. 15.7 Experiment on tracheal regeneration using tracheal prosthesis and autologous chondrocytes [14]. *: Cut end of the tracheal defect area, *black arrow*: recipient site of the tracheal prosthesis, *triangle*: blood vessel. (**a**) Schematic diagram of the experiment: Chondrocytes were harvested from costal cartilage of a rabbit and cultured for 2 weeks. Subsequently, they were placed in a collagen solution and seeded into a collagen sponge of tracheal prosthesis for autologous tissue replacement. The prosthesis was placed in a culture fluid for 30 min, and the resulting prosthesis was implanted into a tracheal defect of a rabbit. (**b**, **c**) Fourteen weeks after implantation, cartilage was regenerated in the recipient site of the prosthesis. Cartilage was stained with Alcian blue. In addition, there was angiogenesis in the cartilage

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Part VII
Future Perspective

Chapter 16
Future Prospective

Shigeru Hirano and Shin-ichi Kanemaru

Abstract All human beings have the dream to one day completely regenerate damaged tissues and organs. The past three decades have seen the emergence and development of advanced tissue engineering and regenerative medicine and the great potential now exists to make this dream a reality. However, forecasting the future direction of tissue engineering and regenerative medicine is no simple matter. Within a span of less than 10 years, induced pluripotent stem cells (iPSCs) were established and the three-dimensional bioprinter (3D bioprinter) were developed. These innovations that seemed beyond the scope of our imagination have the potential to make remarkable progress and dramatic shifts in the medical field.

For simple tissues, such as skin and blood vessels, these innovations have achieved some marked success. However, there are major issues that limit the application of these strategies to complex tissues and organs. It is unreasonable to expect that simple implantation of organs created ex vivo into the living body will result in successful regeneration. Blood supply is an absolute requirement for engraftment into the host and innervation is also necessary for functional regeneration. The key to complete successful regeneration lies in the appropriate combination of cells, scaffolds, and regulatory factors, and critical to this is the creation of a favorable regenerative environment.

The otolaryngology, head and neck field, encompasses regions that consist of various organs and tissues that are critical for maintaining important functions such as mastication, articulation, phonation, respiration, and swallowing. These body systems also have several important neurosensory functions including hearing, balance, smell, and taste. Once these functions have deteriorated, quality of life (QOL) is greatly compromised. This field represents one of the best opportunities where tissue engineering and regenerative medicine is expected to work as an innovative

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strategy. However, it is important to translate successful results from the laboratory into clinical application. The development of iPSCs was one breakthrough that changed the general principle of regenerative medicine and has a great potential to advance this field. When considering the future directions, we must evaluate the current state of technology and potential trajectory in terms of clinical application and advancement of new concepts and strategies.

Keywords Regeneration • Head and neck • Future prospective • Clinical application

16.1 Clinical Application

Applying tissue engineering to human subjects is no simple matter, but the translation of these technologies from laboratory achievement into innovative treatments for human patients is vitally important.

16.1.1 iPSCs

The invention of iPSCs by Yamanaka and Takahashi et al. in 2006 was a breakthrough in the area of regenerative medicine, and completely changed the notion that differentiated somatic cells cannot be reprogrammed. With this technique, new strategies for regenerative medicine have been made possible, including disease-specific modeling, drug screening, and drug development.

While a regenerative approach using iPSCs may seem most promising, serious considerations must be taken with iPSCs given their considerable potential to create tumors. However, some attempts have been made to apply iPSC technology to tissue regeneration. Takahashi et al. successfully created retinal pigment epithelial cells from autologous iPSCs [1]. This was the first attempted clinical application of iPSCs, targeting age-related macular degeneration. A phase I clinical trial is still ongoing in human patients.

In regard to the head and neck region, Imaizumi et al. [2] have conducted animal studies for regeneration of the trachea. They implanted iPSCs along with a scaffold to tracheal defects in rats and were able to confirm regeneration of the trachea in two of five rats. However, there was significant formation of teratomas observed. They also successfully induced epithelial cells of the vocal fold mucosa in vitro from iPSCs [3]. However, the potential for clinical application is still uncertain until the issue of tumorigenesis can be resolved.

A more promising usage of iPSCs could be in drug development and the use of disease-specific iPS cell lines. The use of human iPSCs allows quick, accurate, and efficient evaluation of new drugs in terms of their therapeutic effects and adverse effects. Yahata has established an Alzheimer's disease model from iPSCs which express

amyloid-beta, the causative agent of the disease, and the development of a new drug screening platform using this system is anticipated [4]. The development of disease-specific iPS cell lines is warranted for the field of otolaryngology, e.g., for the treatment of inner ear diseases and fibrotic diseases such as vocal fold sulcus, among others.

16.1.2 Autologous Stem Cells

Autologous stem cells pose no risk of immune rejection, which makes them most suitable for clinical application. Indeed, several human clinical trials have already been conducted or are currently under way using autologous stem cells. Bone marrow–derived mesenchymal stem cells (BM-MSCs) and adipose-derived mesenchymal stem cells (ASCs) have been shown the most interest because they are easy to harvest and proliferate in a culture dish. These stem cells are indeed promising, and numerous studies have been undertaken to confirm their ability to regenerate several organs. Human clinical trials using BM-MSCs have also been performed for patients with conditions such as maxillofacial defects, arthritic lesions, femoral osteonecrosis, and others. There are also many ongoing clinical trials using these stem cells.

BM-MSCs and ASCs have been used for vocal fold regeneration in animal studies and have proven useful for regenerating scarred vocal folds by local transplantation [5–9]. Both types of stem cells have similar biological activity, but some investigators prefer ASCs because they are easier to harvest and have a greater ability to secrete growth factors such as hepatocyte growth factor (HGF). ASCs can be harvested at a ratio of a single cell per 100 cells, while BM-MSCs can only be collected as a single cell per 10^5 cells. We have conducted a comparative study in which scarred rat vocal folds were treated with a local injection of either BM-MSCs or ASCs. The results showed similar effects on recovery of hyaluronic acid in the vocal fold lamina propria, but ASCs showed greater ability to produce hyaluronic acid synthase and HGF [10].

While the technology seems poised to use BM-MSCs or ASCs in human subjects, there are strict regulations and guidelines which govern the usage of MSCs. These GMP-compatible cells must be prepared at a certified cell processing center (CPC). In order to guarantee the high quality of cells, complete sets of cell surface markers for each cell source are needed. Additionally, full data packages must also be prepared, which certify the effects and safety aspects of the cells. Working within these restrictions, there is a human clinical trial currently being conducted at Karolinska University in Sweden focused on the head and neck region using BM-MSCs for the treatment of vocal fold scarring.

16.2 Exploratory Clinical Trials for Growth Factors

There are many kinds of growth factors that are considered to have a powerful ability to regenerate several organ types throughout the body. However, it is important to select the appropriate growth factor for each organ and tissue. It is also necessary

to determine the appropriate conditions for use, such as concentration, number of administrations, and timing; without these careful considerations, growth factors can cause adverse effects. Local application of growth factors is regarded as relatively safe, but full data sets for safety aspects are required before beginning exploratory clinical trials.

Exploratory clinical trials are in preparation for regeneration of the tympanic membrane and the vocal fold. Basic fibroblast growth factor (bFGF) is a strong stimulant of fibroblasts and also regulates cellular function. Fibroblasts treated with bFGF show increased production of hyaluronic acid (HA) through the upregulation of HA synthase gene expression. Basic FGF also has strong angiogenic effects which lead to increased blood supply and growth factors to the target tissues. Kanemaru et al. reported positive effects of implanted gelatin sponge combined with bFGF on regeneration of the tympanic membrane in patients with tympanic perforation [11]. A global clinical trial is currently under preparation to further test this in Japan and the United States.

Hepatocyte growth factor (HGF) has strong anti-fibrotic activity and several animal studies have demonstrated positive effects of HGF on vocal fold regeneration for vocal fold scarring. HGF also induces angiogenesis, upregulates expression of HA synthase and MMP, as well as downregulates collagen gene expression in fibroblasts. These effects indicate the presence of ideal conditions for resolving scar tissue and regenerating tissues such as the vocal fold. GMP-compatible HGF has been developed in Japan as a 5-amino acid deleted variant of HGF (dHGF), and is suitable for use in humans [12]. We have confirmed the beneficial effects of dHGF on vocal fold scarring in canine models [13] and also confirmed the safety aspects of local injection of dHGF into the vocal fold in terms of local adverse effects such as edema or erythema, migration to the blood vessels, and local retention [14]. Based on this data set, an exploratory phase I/II clinical trial is now in preparation to explore the effects and safety aspects of HGF for patients with vocal fold scarring and sulcus.

These types of exploratory clinical trials are necessary for the development of new drugs and to obtain subsequent FDA approval.

16.3 Innovations in Decellularized Scaffolds

Decellularization techniques allow acellular scaffolds to be obtained without the risk of immune rejection. Recent advances in this technology have resulted in the production of scaffolds using several extracellular matrix (ECM) components and growth factors. Urinary bladder-derived material (UBM) has been made available by chemically removing cells, while preserving ECM and growth factors including VEGF and FGF, among others [15]. This type of complex material also preserves vessels and may be the ideal scaffolding for inducing regeneration of several organs. Indeed, the regenerative advantages have been demonstrated in many organs including the esophagus, heart, and eardrum. We examined the regenerative effects of UBM for post-hemilaryngectomy defects by implanting UBM onto the defect

site in a canine model. The results showed promising potential for simultaneous regeneration of cartilage, muscle, and the vocal fold mucosa [16]. This technology may represent a significant breakthrough in "in situ tissue engineering". Recently, the clinical application of UBM has been reported for skeletal muscle regeneration in human subjects with volumetric muscle loss (VMI); promising results were observed in the regeneration of muscular tissue that expressed desmin, an early protein marker of muscle cells [17].

Porcine small intestine submucosa (SIS) is another decellularized scaffolding material that has similar characteristics to UBM [18]. Many animal studies have confirmed the regenerative effects of SIS on muscle, esophagus, bladder, tendon, and others. Commercially available forms of SIS have been developed: Restore™, a ten layer configuration of SIS, or CuffPatch™, another ten layer configuration of porcine SIS that uses a different cross-linking agent. The clinical application of SIS has been reported for the regeneration of abdominal defects [19].

While these decellularized ECM scaffolds have proven to have considerable regenerative effects when used independently as scaffolding, it is possible that these scaffolds can also be utilized with the addition of seeded cells. Decellularized scaffolds have the potential to become a significant tool for use in the field of otolaryngology.

16.4 3D Bioprinting

A 3D bioprinter is a new device for fabricating biological tissues/organs by dispensing cells onto a biocompatible scaffold. This makes it possible to spatially regulate the application of cells and morphogens onto a 3D scaffold. This represents a strong tool for fabricating tissues/organs that mimic native anatomical structures.

Tissues/organs are composed of multiple cells distributed around an extracellular matrix. Early stages of technological development attempted to create biocompatible composite scaffolds using a 3D printer [20]. Cell proliferation and inward growth onto the scaffolds were observed in ex vivo culture. With the present stage of technology, multiple cell types can be delivered layer by layer to predetermined locations on a composite scaffold located in a chamber under an inkjet bioprinter [21]. In some reports, tissue spheroids composed of different cell types were robotically placed into 3D-printed alginate molds using a 3D printer, and these were found to rapidly fuse into toroid-shaped tissue units [22].

In the near future, it will be possible to use a 3D bioprinter to create relatively simple tissues/organs for use in the otolaryngology, head and neck field, such as a trachea or mandible mainly composed of cartilage or bone [23, 24]. A 3D bioprinter can be equipped with multi-printheads for simultaneous injection of several types of cells, scaffolding such as a hydrogel, collagen, or other biomaterials as well as growth factors. A 3D model for printing is designed using images captured from a CT scan or MRI of the patient. With this type of technology, the possibility exists to target treatments for numerous diseases, such as defects in facial/temporal/cranial bone or trachea caused by cancer surgery, injury, or congenital disease.

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