博士論文

Intra- and extra-cellular signaling between dermal papilla cells and hair follicle epithelial cells

(毛乳頭細胞と毛包上皮細胞間の細胞内・細胞外情報伝達)

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September 2022

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CHAPTER 1

1. Introduction

1.1. Brief description and importance of hair

The skin covers the surface of the human body and is the largest organ in the body. As the first line of defense, the skin protects us from the invasion of external adverse factors. From outside to inside, the skin can be divided into epidermis, dermis and subcutaneous tissue. The skin also includes accessory organs, such as hair, sweat glands, sebaceous glands and nails of fingers or toes. Among them, hair is a very important part of skin, for most mammals. Hair covers the surface of the skin and acts to protect the skin, reduces the loss of heat, and keeps warm under certain conditions. For humans, the most prominent hair covers the scalp, greatly affecting people's appearance.

Maslow's Hierarchy of Needs is an important theory in psychology that can be depicted as a hierarchy within a pyramid. From the bottom up, it can usually be divided into five levels, namely physiological needs (food and clothing), safety needs (job security), social needs (friendship), esteem needs and self-actualization [1]. Maslow believed that low-level needs are directly related to the survival of individuals, also known as deficiency needs. When this kind of need is not met, it is directly life-threatening. Moreover, high-level needs are not absolutely necessary to maintain individual survival. However, satisfying this kind of needs makes people live healthier, longer, and more energetic, and high-level needs are called growth needs. High-level needs are more complex than low-level needs, and low-level needs must be satisfied before high-level needs appear. In the evolution from other animals to humans, higher needs appeared relatively late. Babies have physiological and safety needs, but self-actualization needs emerge after adulthood. All living things need food and water, but only humans have self-actualization needs [1].The pursuit of beautiful appearance takes precedence over lower-level needs

(physiological and safety needs) and contributes to social needs, respect from or to others, and self-actualization. The pursuit of beauty makes human beings happier, more energetic, and makes life more colorful. In the pursuit of beauty in appearance, the beauty of hair is one of the beauty methods that has been continuously developed through the ages. People can add accessories to the hair or change the color and curl of the hair for aesthetic purposes. The appearance and functional integrity of hair is also gaining more and more attention.

1.2. Hair growth cycle and important cells involved

Hair growth is periodic, which is related to the growth cycle of hair follicles (Figure 1). The hair growth cycle includes the anagen, catagen, and telogen phases. The Anagen phase is a growth period, lasting 2–6 years. Under normal circumstances, approximately 90% of hairs are in the anagen phase [2]. During the anagen phase, epithelial cells continue to proliferate to form hair shafts. Telogen is a resting period that lasts approximately 3–5 months following which the hair falls out. Catagen, the transitional period between anagen and telogen, lasts for approximately three weeks. In the catagen phase, all hair growth stops. Less than 1% of hair from the normal scalp are present in this period [2].

The production and maintenance of hair follicles are inseparable from two kinds of cells (Figure 2), hair follicle stem cells (HFSCs) and dermal papilla cells (DPCs). HFSCs are a type of hair follicle epithelial cells. HFSCs are considered epithelial cell reservoirs that are essential for the maintenance and remodeling of the epithelium. The epithelium plays an important role in the hair growth cycle [4]. Horizontally, the epithelium can be divided into hair shaft, inner root sheath, and outer root sheath from the inside to the outside. Longitudinally, the epithelium is divided into the beginning of the interfollicular epidermis, infundibulum, isthmus, suprabulbar region, and bulb from top to bottom [3].

The isthmus is between the entrance of the sebaceous duct and the attachment area of the arrector pili muscle. The isthmus is essential because it includes the so-called "bulge" area in which the HFSCs are located. Besides, the outer root sheath layer at the proximal end of the isthmus is defined as a bulge as well, and HFSCs are also present here [4]. Mouse models have confirmed that stem cells from hair follicles have the ability to rebuild the damaged interfollicular epidermis and play a role in repair [5][6]. When necessary, HFSCs migrate down to the hair matrix area, where they transform into progenitor cells, and then form internal hair follicles and hair shafts [7]. The dermal papilla (DP) at the bottom of the hair follicle originates from the mesenchyme, and DPCs are different from epithelium of hair follicle in morphology and structure. DPCs are key regulators and inducers of the formation of new hair follicles and even entire hair growth via paracrine secretion [8]. DPCs maintain the growth of the epithelium of hair follicles, whereas the epithelium needs the help of DPCs to be organized into complex hair structures [9]. During the transition from the late telogen phase to the early anagen phase, signals from DP stimulate the hair germ and activate the resting bulge stem cells [10]. During anagen, cells at the bottom of the hair follicles begin to proliferate to form new hair filament, and the stem cells in the bulge produce hair germs. Although apoptosis occurs in hair follicles in catagen and telogen, DP remains intact and migrates upwards until it is close to the bulge.

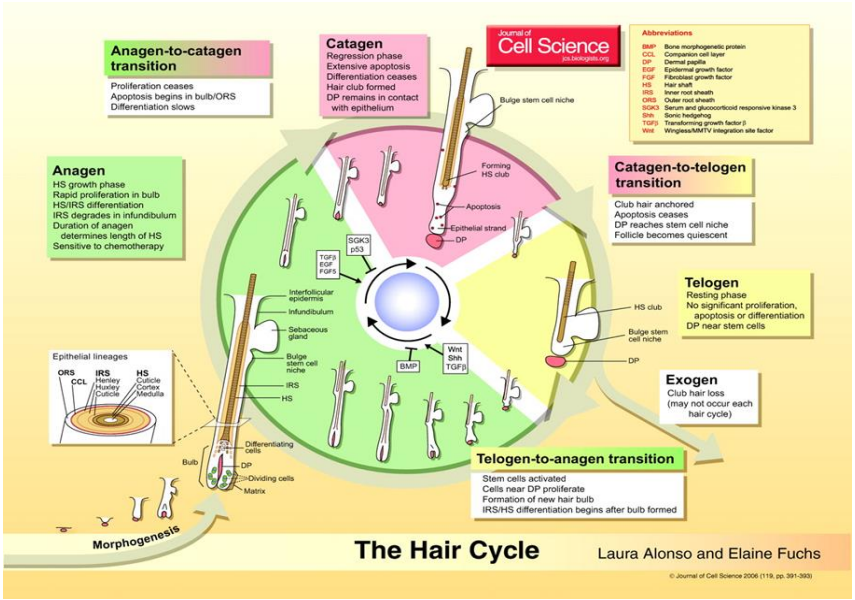

Figure 1. The hair growth cycle [11]. The hair cycle is typically divided into anagen, catagen and telogen. Exogen is an extension or part of the telogen. 70–100 hairs fall out every day when brushing or shampooing in the exogen phase.

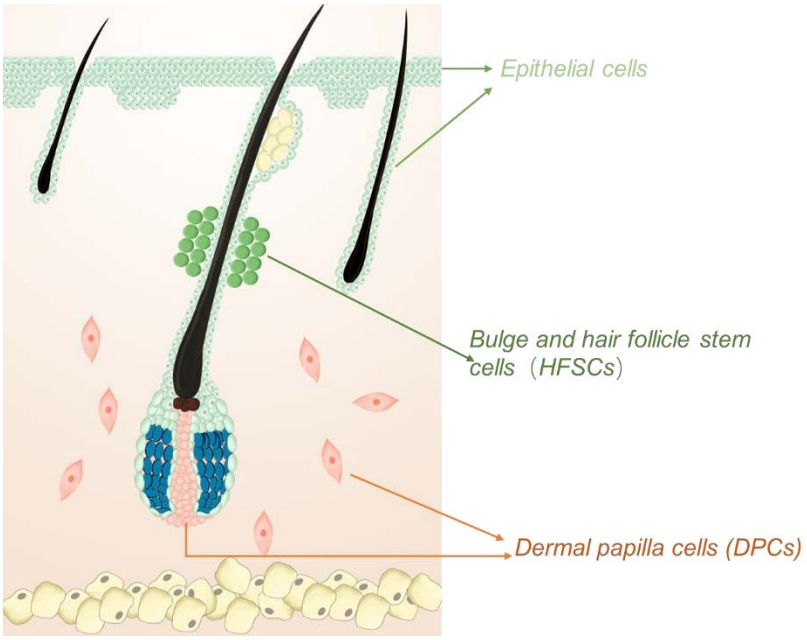

Figure 2. Basic structure of the hair follicle

1.3. Hair loss

In daily dermatology clinic, doctors often see patients with hair diseases. Among hair disorders, hair loss is the most common. Hair loss is divided into many types according to different pathogenic factors. For instance, diseases such as woolly hair, monilethrix, atrichia, and hypotrichosis are often genetically related. Among the pathogenic factors of androgenetic alopecia (AGA), genetic factors are also the main reason. Hair loss occurs as a symptom of systemic diseases, such as thyroid disease or insulin resistance. Some types of hair loss may be related to the body's autoimmune state, for example, alopecia areata or lupus erythematosus may occur when the autoimmune regulation is unbalanced. Sometimes nutritional disorders cause hair loss. Excluding the lack of nutritional intake caused by economic factors, this type of hair loss in modern society is seen in some people who are on diet. Certain environmental factors (such as radiation) or aging also cause hair loss. The occurrence of trichotillomania (a disease that causes hair loss by pulling hair

spontaneously) is related to the patient's nervousness or stress [12].

1.3.1. Manifestation and onset of androgenetic alopecia

Among many types of hair loss, AGA has the highest prevalence. AGA is common in male patients, although female patients also suffer from this disease. The disease can be divided into two patterns according to its appearance, that is male pattern and female pattern. In the male pattern hair loss, the hairline regresses at the bitemporal regions, resulting in vertex baldness. The mildest manifestation is that there is only a bilateral temporal hairline receding similar to cat ears, and the most serious manifestation is that the overall scalp only has a circle of hair around the back occipital and ear temples. In the female pattern hair loss, the hair is diffusely thinned in a larger area, whereas the forehead hairline is preserved [13]. Male patients are prone to male pattern hair loss, while female patients are prone to female pattern hair loss, but it does not mean that male patients do not suffer from female pattern hair loss or female patients do not suffer from male pattern hair loss. AGA affects the appearance of patients, and causes a certain level of mental stress. Some patients may even lose confidence, which affects their lives or work. One study [14] used questionnaires and scales to quantify the impact of hair loss on quality of life, where hair loss was not limited to AGA. The questionnaire consisted of the original Dermatology Life Quality Index (DLQI) questionnaire (10 questions specifically designed to assess the quality of life of patients with dermatology) and some adapted questions on the effects of hair loss. An abbreviated version of the Center for Epidemiologic Studies Depression Scale (CES-D)12 was also used. This scale has a maximum score of 30, with a score greater than 8 indicating possible depression. Results have shown that DLQI scores of alopecia responders were similar to patients with severe psoriasis, which is a chronic skin disease characterized by red papules or plaques covered with silvery-white scales that can severely impact quality of life. 74% of patients had a

CES-D score greater than 8, indicating that they may have clinical depression. After the initial event, hair loss continued to have a significant impact on quality of life, with 40% of patients also dissatisfied with the way doctors treated them. This study confirmed that people with hair loss might lose self-confidence and increase self-awareness.

The AGA patient's age of onset is usually 30 and 40 years old. Some patients have been observed to begin to lose their hair after puberty and gradually get worse [15]. A total of 954 patients (535 women, 419 men) were included in a study by Salman et al. [16]. Among them, the prevalence of AGA was 67.1% in men and 23.9% in women. The prevalence and severity of AGA were related to the age in both sexes (p=0,0001). A study by Paik et al. [17] investigated 10,132 Koreans (5531 men and 4601 women) and analyzed the prevalence and types of AGA. The prevalence rate of male patients of all ages was 14.1%. Divided by age group, the prevalence rate was 2.3% in the 30s; 4.0% in the 40s; 10.8% in the 50s; 24.5% in the 60s; 34. 3% in the 70s; 46.9% over 70 years old. The "female pattern" was observed in 11.1% of male cases. The prevalence of AGA in female patients of all ages was 5.6%. Among them, the prevalence rate was 0.2% in the 30s; 2.3% in the 40s; 3.8% in the 50s; 7.4% in the 60s; 11.7% in the 70s and 24.7% over 70 years old. Besides, 48.5% of male patients and 45.2% of female patients had a family history of baldness.

Clinically, some grading methods (Figure 3) are used to measure the severity of AGA. For male pattern hair loss, the Hamilton-Norwood scale [18] is used, while the Ludwig scale [13] is commonly used for female pattern hair loss.

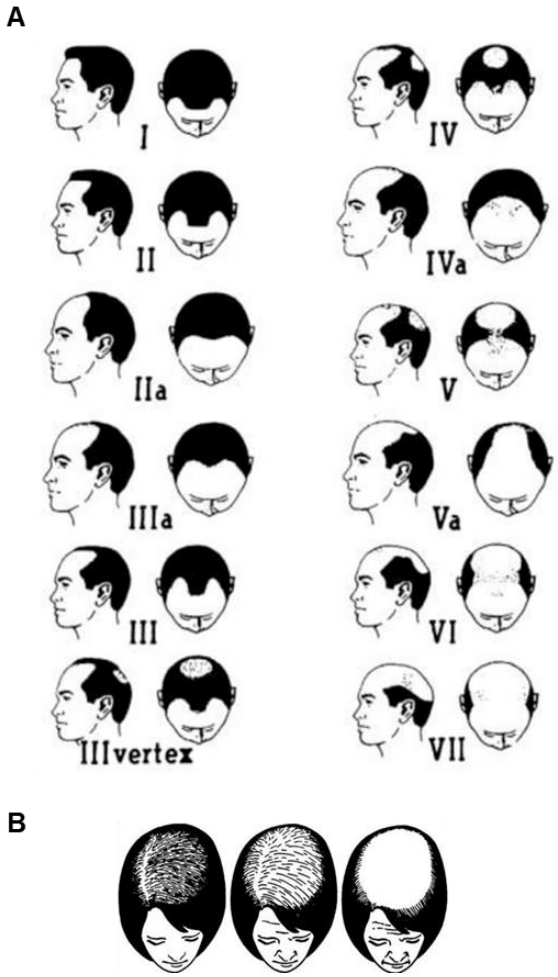

Figure 3. Two grading methods for AGA. (A) The Hamilton-Norwood scale [18]. (B) Ludwig scale [13].

Figure 4. A typical AGA patient. The patient was a Chinese male, 37 years old, had a 6-year course of AGA, and was classified as Hamilton–Norwood scale III. The photos were from this patient who was treated by the author in 2017. Informed consent for research

use of the photos was obtained from the patient.

1.3.2. Causes of androgenetic alopecia

The occurrence of AGA is related to genetic factors. Clinically when asking about the medical history of male patients, it is often heard that their paternal relatives also suffer from the disease. A study by Nyholt et al. [19] investigated male pattern hair loss in 476 monozygotic and 408 dizygotic male twin pairs, and found that the heritability rate was 0.81 (95% CI: 0.77–0.85), thus confirming that genetic effects play a major role. Genome-wide association studies (GWAS) suggest that the biology of AGA is less complex than other human traits, such as height or body mass index [20]. The GWAS of the AGA have identified 12 genomic regions and some candidate genes. Candidates include genes encoding androgen receptor (AR), histone deacetylases (HDAC) 4, HDAC9, WNT10A, and so on [21]. The most closely related genomic regions are located on the long arm of the X chromosome (Xq). Both sides of this region encode the AR and the ectodysplasin A2 receptor (EDA2R). Given the androgen dependence of the AGA phenotype, AR is considered the most plausible candidate gene for AGA. A second candidate gene in this region is EDA2R, which is associated with anagen maintenance [22]. Notably, in males, the X chromosome is inherited from the mother. The remaining 11 associated genomic regions are located on autosomes. Clinically, similarities can be observed in the pattern of hair loss between AGA-affected fathers and their sons. This similarity is conferred either by genetic variation on the Y chromosome, which is inherited from the father but contains few genes, or by autosomal genetic variation, which is inherited to the same degree from the father and mother. The latter contains the vast majority of human genes, including some that have been associated with AGA [23]. On autosomes, the greatest association with AGA is the intergenic region on chromosome 20 between paired box 1 (PAX1) and forkhead box A2 (FOXA2). Besides, HDAC4 on autosome 2q37 and HDAC9 on autosome 7p21.1 have been found to be expressed in human hair follicles

[24]. HDAC4 and HDAC9 regulate AR signaling through interactions with AR proteins [24][25]. Therefore, both HDAC and AR proteins may contribute to androgen-induced hair follicle miniaturization in AGA. In addition, the AGA-related genes contain two members of the TWIST basic helix-loop-helix (bHLH) family of transcription factors, namely TWIST2 (2q37) and TWIST1 (7p21.1). Experiments in adult mouse DPCs have shown that TWIST1 is involved in the hair growth cycle regulation, which acts to regulate the transition from anagen to catagen [26]. TWIST2 has been implicated in the development of the mesenchymal cell lineage [27]. There may be functional interactions between HDAC4, HDAC9, TWIST1, and TWIST2 during AGA development, which may lead to androgen-induced follicle miniaturization, shortened anagen and premature catagen entry [23]. Another important region is autosome 2q35, where the most relevant is rs7349332, located in WNT10A. WNT10A is expressed in human hair follicles, and has specific roles in anagen induction and maintenance. Genetic risk alleles at this locus may lead to the appearance of premature catagen of hair follicles or delayed entry of catagen into anagen [28].

AGA is also understood to be a phenomenon in which the hair growth cycle becomes extremely short because of the influence of androgen and so that the hair falls out before it grows sufficiently. In the hair follicles of the scalp of genetically susceptible individuals, circulating testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase. DHT induces apoptosis in DPCs [29]. It also inhibits the Wnt/β-catenin pathway, which is important for the initiation and maintenance of anagen phase [30][31][32]. Therefore, DHT and activated androgen receptors cause the anagen phase to be shortened, which leads to the miniaturization of hair follicles, making the hair follicles thinner and shorter. Eventually hair follicles may not even penetrate the epidermis [33][34]. Generally, under the influence of androgen, terminal hair was turned into vellus hair [35]. Terminal and vellus hair are the two classifications of hair morphology. Terminal hair is over 0.03 mm in diameter and over 1.0 cm in length that may be pigmented and medullary. Terminal hair is rooted deep in the subcutaneous tissue or dermis. Vellus hair refers to hair less than

or equal to 0.03 mm in diameter, less than 1.0 cm in length and lacks pigment and medulla. The vellus hair is rooted in the upper part of the dermis. When the terminal hair is miniaturized to vellus hair, it is called vellus-like hair [36]. Under dermoscopy, one of the major manifestations of male pattern baldness is an increase in vellus hair and a decrease in terminal hair. The proportion of vellus-like hair follicles also increases.

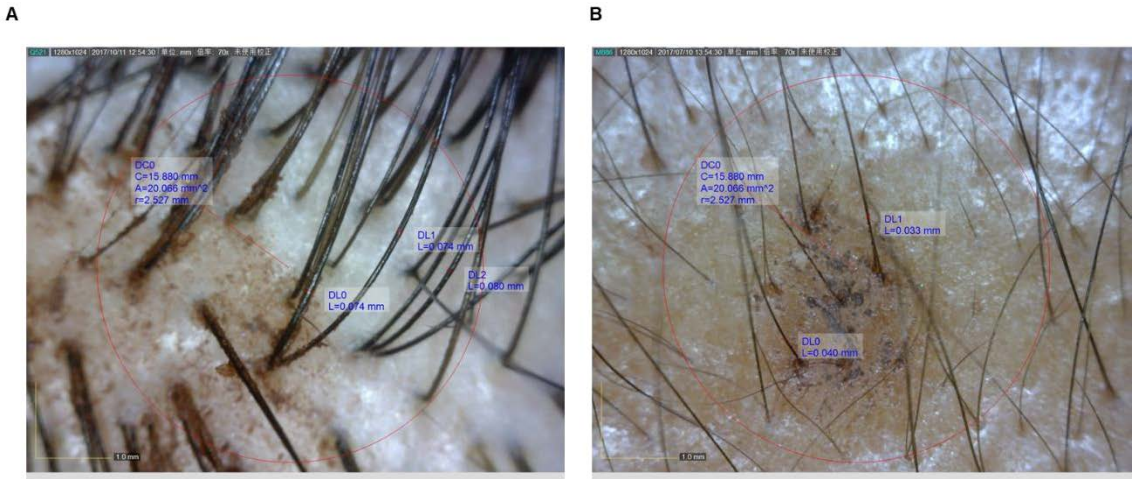

Figure 5. Photos of terminal hair and vellus hair taken by Dino-Lite Digital Microscope. These two photos are from one patient the author once treated. (A) The Photo from the non-alopecia area, which looked normal. (B) The photo from the alopecia area.

1.4. Treatment for hair loss

1.4.1. Drug treatment

The current main drug treatments are oral finasteride and topical minoxidil. Finasteride, a 5-alpha reductase inhibitor, inhibits the conversion of testosterone to DHT by blocking type 2 5-alpha reductase [37]. In males, the side effects of finasteride treatment include decreased libido, erectile dysfunction (ED), and ejaculatory disorders. The use of finasteride by women is relatively limited. If women of childbearing age use finasteride, there is a risk of feminization of the male fetus [38]. Women's use of finasteride may also lead to a relative excess of estrogen or androgen deficiency, which is related to an

increased risk of breast cancer [39]. Drugs with similar pharmacological effects include dutasteride, which is an inhibitor that blocks the activity of both type 1 and type 2 -alpha reductase. But in most countries, it is still an off-label drug. Minoxidil appeared in the 1970s as a treatment for hypertension. However, in patients treated for hypertension, 24–100% of patients were observed to have the side effect of hair growth [40]. There are currently 2% solution, 5% solution and 5% foam on the market. Minoxidil can treat hair loss because it promotes hair growth by opening potassium channels, leading to increased blood flow and levels of vascular endothelial growth factor in the DP [41]. The common side effects of minoxidil include facial hirsutism and allergies. When using minoxidil, the appearance of increased hair on the facial skin near the scalp is observed. Allergy may be caused by propylene glycol, the ingredient in minoxidil solution, so the adverse reactions of 5% solution are more common. Because the foam minoxidil does not contain propylene glycol, it is suitable for people with allergic reactions [42]. Whether it is oral or topical drugs, long-term treatment is required for months, years, or even decades, and there is no upper limit of time. Patients with poor adherence may not be able to persist.

1.4.2. Hair transplantation

In 1931, Okuda [43] was the first doctor to describe the technique of hair transplantation. He explained how to use a circular punch to get the graft from the scalp and insert it into a slightly smaller hole in the hair loss area of the scalp. In 1959, Orentreich [44] proposed the term "donor dominance" to refer to a graft that retains its original characteristics after being transplanted from a donor area to a recipient area. This concept is very important and instructive. In hair transplantation, the hair follicles in the occipital region insensitive to androgen can be transplanted to the scalp in the androgen-dependent region, and properties of these hair follicles are preserved. In the mid-1990s, follicular unit transplantation (FUT) emerged. Strips of tissue are excised from the

occipital scalp and then microscopically isolated into individual follicular units (FUs), which are implanted into holes in the recipient area. The FU is the main structure used in hair transplantation. Human hair occurs on the scalp in tufts or groups called FUs [45]. The FU contains terminal hair follicles, vellus hair follicles, sebaceous glands, arrector pili muscles, perifollicular dermis, adipose tissue, exocrine coils, nerve and vascular networks [46][47][48]. After the donor site is sutured, a slender, linear scar will appear. Except for the patients with very short hair, most of the scars can be covered by the hair. In the early 2000s, follicular unit excision (FUE) emerged, using a 0.8–1.0 mm punch to individually remove FUs directly from the donor area. This method does not require sutures of the donor site, and results in no linear scar [49]. Regardless of the surgical approach, care must be taken to maintain the anatomical integrity of FUs during transplantation, especially in the bulge and DP areas that are critical for hair growth and maintenance, in order to avoid compromising graft survival.

Hair transplantation is the redistribution of a patient's existing hair from the donor area to the recipient area and therefore does not increase or produce new hair. At the same time, hair transplantation is limited by the number and other conditions of hair follicles in the donor site. For AGA, it's a lifelong process, and as a result, hair transplantation doesn't change the progression of disease. For patients with progressive hair loss, drug treatment is usually required to stabilize the disease. For some patients, multiple hair transplantation may be required to achieve satisfactory results. Noninflammatory secondary scarring alopecia includes alopecia caused by posttraumatic wounds, surgical scars and burn scars. For these types of hair loss, hair transplantation is very beneficial, as long as there are enough hair follicles in the donor area that can be used to achieve the effect of covering the scar after transplantation [50].

The number of FUs required for hair transplantation depends on the severity of the hair loss, the condition of the donor site and the patient's expectations. As for the relationship between hair density and FU density, in general, the hair density is about 2.5–3.0 times

higher than that of the FU density. Therefore, if 3000 FUs are transplanted, the number of hairs produced is expected to be around 7500–9000 [51]. Typically, a complete transplantation requires multiple surgeons and nurses to work together and can last for hours. In addition, as a surgery, hair transplantation is relatively expensive.

1.4.3. Cell therapy

In recent years, research on hair loss treatment has begun to focus on the cellular level. Researchers work on methods to rejuvenate the cells that enable hair growth. Among them, the most popular topic is hair regenerative medicine. Hair regenerative medicine is a treatment that effectively utilizes the function of cells to regenerate tissues and organs for hair follicles which have been weakened or damaged by diseases.

DPCs are key regulators and inducers of new hair follicle formation and even overall hair growth, and turn to be targets of research. In the study by Jahoda et al. [52], a cell suspension containing epithelial cells and mesenchymal cells with hair-inducing ability (DPCs) was injected into the skin of immunodeficient and hairless mice. Hair regrowth was observed, and they also found that serially cultured DPCs can induce hair growth when implanted into follicles. This approach is the basic principle of current hair regeneration medicine, that is, the spontaneous regeneration of hair follicles by transplanting a combination of epithelial and mesenchymal cells.

Many methods have been investigated to maintain or restore the properties of DPCs. Among them, spheroid culture is used as a method of three-dimensionally constructing cells in a near-living environment. Compared with the traditional monolayer culture, the spheroid culture can maintain cell function for a long time and improve hair regeneration ability [53]. Common methods for DPC spheroid preparation include hanging drops, non-cell adhesive dishes and spheroid microarray devices, which were observed to produce higher hair inductivity than that in a two-dimensional (2D) environment [54][55][56].

1.4.4. Other treatment

Platelet-rich plasma (PRP) and low-level laser therapy (LLLT) can also be used to treat hair loss. PRP treatment is mainly to extract the patient's own venous blood, followed by one or more centrifugations under sterile conditions, although two centrifugations are most common. Activated PRP is injected into the patient's scalp subcutaneously. Three injections of PRP are recommended because the effect of PRP seems to peak after 3 to 5 injections [57]. The research topic of the author of this article during the master's degree was PRP treatment of male AGA, using the plan of once a month, a total of 3 times of injection. Although PRP injection is not an FDA-approved treatment for hair loss, it is clinically effective to a certain degree. PRP can be considered for patients for whom conventional therapy is not appropriate. In addition, PRP can also be used as a supplement to other conventional treatment such as finasteride and minoxidil. However, PRP is an invasive treatment, and some patients cannot tolerate the pain of the injection. At the same time, some hospitals or institutions have many restrictions on the management of blood products.

Red or near-infrared lasers have the ability to promote tissue repair and regeneration, where low-intensity light from LLLT stimulates cellular activity [58]. In the late 1960s, a study [59] irradiated mice with a low-power ruby laser (694 nm) to see the carcinogenic potential of the laser. However, interestingly the laser did not cause cancer, but stimulated hair growth in shaved mice. Since then, the role of LLLT in promoting hair growth has attracted public attention. At present, most studies have used wavelengths in the 635 to 650 nm range. Devices currently available on the market include combs and helmets. Typically, 10–30 minutes of irradiation 2–7 times per week is clinically effective [59]. As a commercially available device, patient adherence is very important in the treatment of LLLT. At the same time, more clinical research is needed to prove its efficacy and safety.

Although the disease of hair loss is not fatal, it seriously affects the life and work of modern people. More effective and more acceptable treatment methods are constantly

being developed and researched.

1.5. Exosomes

The first discovery of exosomes was in the 1940s. However, at that time, they were thought to function in disposal because they removed unwanted cellular components [60]. In the 2000s, the role of exosomes was gradually discovered and paid attention to. In 2017, a study reported that exosomes derived from mouse and human mast cell lines and primary bone marrow-derived mouse mast cells contained RNA and microRNA, transferable to another cells. After exosomes entered new cells, their RNAs were translated to produce new proteins [61]. Increasing evidence has shown that exosomes can act as messengers that transmit biological information to distant cells or tissues [60]. At present, in addition to basic research, exosomes have been researched in various medical fields, including immunology, oncology, neurology, cardiovascular and dermatology. The research directions of exosomes are also diverse, including tumor body fluid diagnosis, drug loading ability, anti-infection ability, and tissue regeneration ability. A search of the articles on exosomes (except reviews, books, and meta-analyses) from 2003 to March 2022 on the Pubmed webpage yielded 101 results. Among them, there are two peaks, that is, 13 articles were published in 2014, and 19 articles were published in 2020. It can be initially seen that in recent years, exosomes have indeed received a lot of attention.

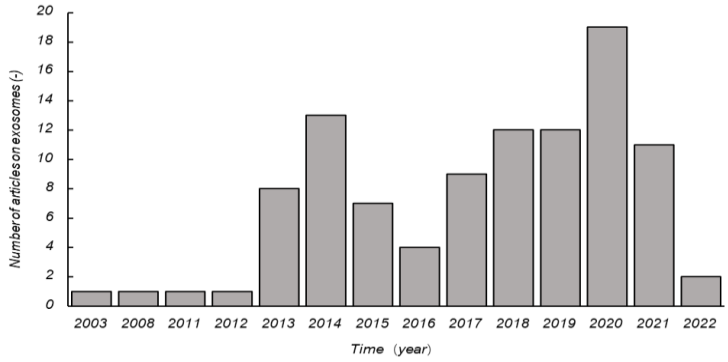

Figure 6. Number of articles on exosomes. The keyword “exosomes” was searched on the Pubmed web page, the article type was defined as clinical trial or randomized controlled trial, and the results of the year and the number of articles were obtained.

Exosomes are belonged to extracellular vesicles (EV), which are lipid bilayer vesicles produced by cells. EVs can be secreted by most types of cells, from bacteria, animal cells to plant cells, which indicates the existence of conservative evolution [62]. In addition to exosomes, EVs also include microvesicles and apoptotic bodies. In terms of size, the diameter of exosomes ranges from 30 to 200 nm; the size of microvesicles is 100 to 1000 nm; the largest is the apoptotic body, which has a diameter from 500 to 2000 nm [60]. From the point of view of formation mechanism, the process of exosomes formation is the most complicated. Exosomes begin to be formed by the inward budding of the plasma membrane. Then, the inward budding of the early endosomal membrane results in multivesicular bodies (MVBs). Finally, the fusion of the plasma membrane with MVBs allows the release of exosomes outside of the cells. Whereas the microvesicles are simply produced by budding out of the plasma membrane [60]. As for the apoptotic bodies, they are the result of death of apoptotic cells and usually contain cell fragments [63].

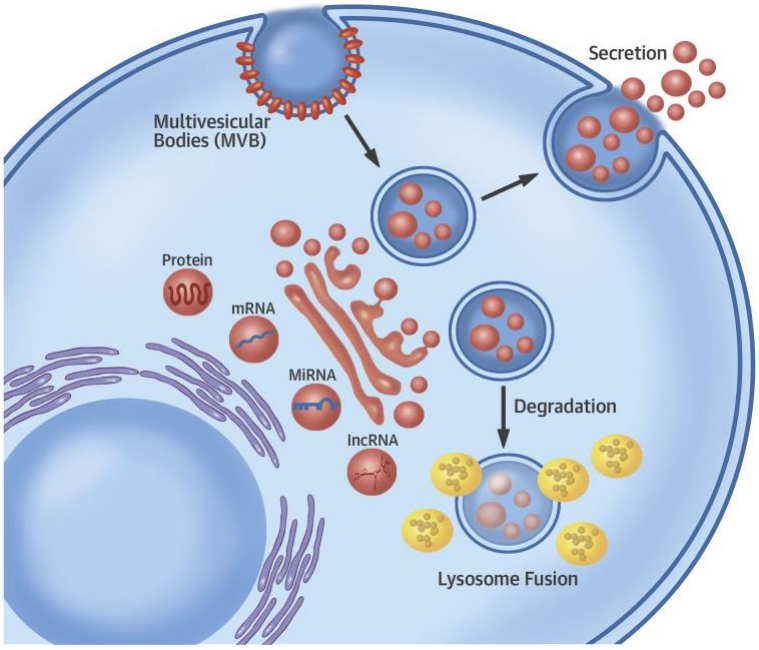

Figure 7. Exosome biology [64].

One study by Jiang et al. [65] indicated that exosomes might mediate epithelial–mesenchymal crosstalk during organ development, showing that exosomes or the

molecular contents they transport may be involved in treating diseases or tissue regeneration. The generation of hair follicles and the maintenance of their morphology and function result from epithelial–mesenchymal crosstalk. Therefore, it can be assumed that exosomes positively affect hair follicles. Exosomes were also reported to play a major role in paracrine signaling. A review suggested that mesenchymal stem cells (MSCs) encapsulated functional proteins or regulatory RNAs in exosomes. The phospholipid layer of exosomes created a rapid intracellular conduction pathway, allowing MSCs to respond early to stimuli [66]. A study by Zhou et al. [67] found that injection of DPC-Exos (exosomes derived from human DPCs) accelerated the onset of anagen of mouse hair follicles as well as delayed the catagen, indicating the delay of transition from anagen to catagen. After DPC-Exos treatment, the expression of β-catenin and Sonic hedgehog (Shh) levels in the skin also increased. β-catenin and Shh signalings are essential for the regulation of hair follicle growth. The Wnt/β-catenin signaling regulates the induction activity between hair follicle epithelium and mesenchyme, while the Shh signaling is a prerequisite for the paracrine action [31][68]. The article of Zhou et al. [67] revealed the paracrine effect of DPCs on epithelial cells and the important role of exosomes in the paracrine mechanism. In another study by Kwack et al. [69], 3D DP-Exos (exosomes derived from human DPCs using three-dimensional culture) increased IGF-1, KGF and HGF in DPCs. After local injection of 3D DP-Exos to mice, the telogen of hair follicles was induced to enter into the anagen, and the anagen phase was prolonged. In addition, when human DP spheres and epidermal cells from dorsal skin of embryonic mice were transplanted together into female nude mice skin, compared with the control groups, the exosomes-treated spheres promoted hair regeneration more.

1.6. Purpose of this research

As mentioned above, hair loss is a common disease in modern society, and the need for treatment is becoming more and more urgent. The current mainstream treatment methods have their own advantages and disadvantages.

Two types of cells are essential for the development and maintenance of hair follicles, namely DPCs and hair follicle epithelial cells represented by HFSCs. At the same time, in hair regeneration medicine, DPCs are a key research target. It is of great research value to identify potentially important signaling pathways responsible for hair induction by studying the intracellular signaling pathways of DPCs. As a result, in this study, gene expression analysis and other experiments were performed to identify potentially important signaling pathways responsible for hair induction in DPCs. The results of this study contributed to provide better methods for preparing DPCs for use in hair regenerative medicine.

Moreover, studying the extracellular signaling pathways of DPCs and identifying the potentially important mechanisms responsible for hair induction will also be very helpful for the study of the treatment of hair loss. Exosomes are microvesicles that have been discovered and endowed with definition in recent decades. Exosomes can be secreted by a variety of cells and have a certain ability to load and transport substances across membranes, thereby affecting the corresponding cells. Exosomes, as products of cellular secretion, are good targets for the study of extracellular signaling pathways.

To date, although there have been some studies on the effect of exosomes on hair growth, the number is still limited. In addition, there are several articles concerning the hair growth-promoting effect of DPC-derived exosomes, and some mention the 2D culture method and the 3D culture method, but there is little comparison of the effect between the two, let alone different 3D culture methods. Different culture methods bring about different cell growth environments, and different extracellular growth environments have different effects on their secretion. Exosomes originate from the invagination of the plasma membrane, during which some extracellular components and cell membrane proteins are packaged together to further form MVBs. MVBs fuse with the plasma membrane and enter the extracellular environment through paracrine and other means to form exosomes. It can be inferred that different culture methods of cells can also have different effects on the generated exosomes. Therefore, this study used three

culture methods (including 2D culture, 96-well-round-bottom plate culture and PDMS microwell array culture method) to culture cells, aiming to understand the extracellular signaling and communication between epithelial and dermal cells via different exosomes and the mechanism behind them. I also intended to explore the differences in exosomes obtained using different culture methods of DPCs to discover new directions for the treatment of hair loss.

CHAPTER 2

2. Intracellular signaling pathways of dermal papilla cells

2.1. Purpose

The growth of hair follicles is inseparable from DPCs. DPCs are more prone to lose hair-inducing function in traditional 2D culture than in 3D culture. Among different kinds of 3D culture, it has been demonstrated that when DPCs are encapsulated in collagen gel droplets to form hair beads (HBs), efficient hair inductivity and hair follicle regeneration could be observed [70]. However, the mechanisms responsible for activating the hair-inducing function of DPCs remain unclear regardless of the culture method. The aim of this chapter was to identify the underlying intracellular signaling pathways of DPCs responsible for hair induction in order to provide better methods for the preparation of DPCs for use in hair regenerative medicine.

2.2. Materials and Methods

2.2.1. Overview of experimental steps

Human DPCs were prepared in both 2D and HB cultures. Gene expression analysis and gene chip analysis were performed to identify potentially intracellular signaling pathways responsible for hair induction in DPCs. Inhibitor and activator of PI3K/Akt signaling pathway were used to observe differences.

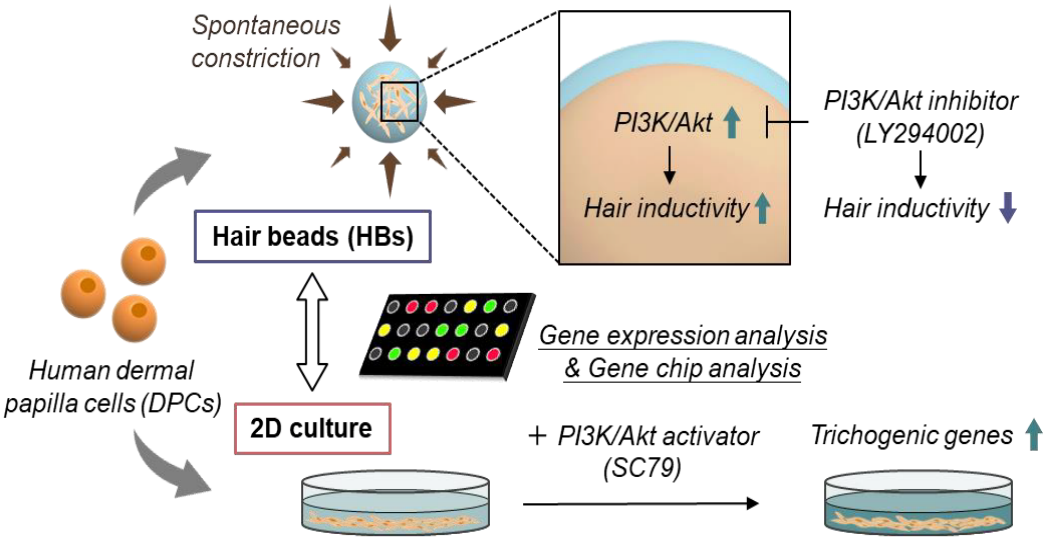

Figure 8. A Schematics of the experimental procedure and steps in this chapter [71]. Transcriptome comparisons of DPCs cultured in 2D and HB cultures after 3 days were conducted to identify important pathways for hair induction. DPCs were exposed to inhibitors and activators of the PI3K/Akt to elucidate the role of this pathway in hair induction.

2.2.2. Culture of dermal papilla cells

Human DPCs at passage 2 (PromoCell GmbH, HDB, Germany) were grown in a culture medium (DPCGM, Follicle Dermal Papilla Cell Basal Medium and Follicle Dermal Papilla Cell Growth Medium SupplementPack; PromoCell GmbH, HDB, Germany). Human DPCs were incubated at 37 °C in 5% CO_2 (CO_2 incubator; PHCbi, Japan). After the cells were approximately 80% adherent, they were passaged by trypsin treatment (0.25% Trypsin-EDTA; Gibco, NY, USA). After the cells grew to cover about 80% of dish surface again, they were suspended in cell banker (CELLBANKER 1; ZENOGEN PHARMA, FKS, Japan) and stored in liquid nitrogen for cryopreservation. DPCs at passage 4 were used for following experiments. As a comparison of HB culture, DPCs (1×10^4 cells/well) were cultured in a 6-well plate (TPP, Japan), which was called

2D culture.

2.2.3. Preparation of HBs

HBs were prepared according to the method by Kageyama et al. [70]. Under the low temperature guarantee of ice packs, a collagen solution was prepared by mixing 0.8 mL of collagen type I-A (Cellmatrix Type I-A; Nitta Gelatin, Japan), 0.1 mL of 10× concentrated Ham's F12 medium (Nitta Gelatin), and 0.1 mL of reconstitution buffer solution (Nitta Gelatin), resulting in collagen solution concentration of 2.4 mg/mL at pH 7. DPCs (1×10^4 cells/HB) were suspended in the collagen solution, followed by being spotted as 2-μL droplets on a flat surface and incubated at 37 °C for 30 minutes for gelation. The microgels, which were named as HBs, were then suspended in DPCGM and cultured in a non-cell-adhesive dish for 3 days, followed by gene expression.

2.2.4. Gene expression analysis

The gene expression of DPCs in 2D culture and HB culture was assessed using real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis after 3 days of culture. Total RNA was extracted using a RNeasy Mini Kit (250) (QIAGEN, Hilden, Germany) and QIAshredder (250) (QIAGEN, Hilden, Germany). All RNA were dissolved in nuclease-free Water. RNA concentration was measured using NanoDrop One (Thermo Fisher Scientific; WI, USA). Nuclease-free water was used to dilute all RNA samples to equal concentration. Reverse transcription was conducted using the ReverTrA ace qPCR RT kit (Toyobo, Osaka, Japan). cDNA was obtained by incubating the samples at 37 °C for 15 minutes and then 95 °C for 5 minutes in QuantStudio 3 (Applied Biosystems by Thermo Fisher Scientific). The cDNA samples were stored at -30 °C until RT-PCR was performed. RT-PCR was conducted with TB Green Premix Ex Taq II (Tli

RNaseH Plus) (Takara Bio, Shiga, Japan) using the corresponding forward and reverse primers (synthesized by FASMAC, Kanagawa, Japan), listed in the following table. Amplification was performed under the following cycling conditions: 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. The $2^{-\Delta\Delta}$ Ct method was used to compare the relative mRNA expression levels of the target genes to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Gene name	**Forward primer 5'-3'**	**Reverse primer 5'-3'**
GAPDH	TGGAAGGACTCATGACCACAG	GGATGATGTTCTGGAGAGCCC
Versican	GGCACAAATTCCAAGGGCAG	TCATGGCCCACACGATTAACA
ALP	ATTGACCACGGGCACCAT	CTCCACCGCCTCATGCA
LEF1	CTTCCTTGGTGAACGAGTCTG	TCTGGATGCTTTCCGTCAT
Wnt5a	TCCACCTTCCTCTTCACACTGA	CGTGGCCAGCATCACATC
PIK3CA	CCCAGGTGGAATGAATGGCT	GCCAATGGACAGTGTTCCTCT
AKT1	GCTCACCCAGTGACAACTCA	CTCAAACTCGTTCATGGTCACG

Table 1. The primer sequences for the RT-PCR analysis of both trichogenic and PI3K/Akt signaling-related genes

2.2.5. Gene chip analysis

After 3 days of culture, total RNA was extracted from DPCs in 2D and HB cultures using a RNeasy Mini Kit (250) (QIAGEN, Hilden, Germany) and QIAshredder (250) (QIAGEN, Hilden, Germany). Gene expression profiling was performed by GeneChip Human Genome U133 Plus 2.0 arrays (Thermo Fisher Scientific). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was conducted by the R package clusterProfiler. The upregulated genes of DPCs in HB culture compared to 2D culture were selected with the threshold of Log2FC > 1.

2.2.6. Inhibition of PI3K/Akt signaling pathway

LY294002 (Abcam, UK) is a PI3K/Akt inhibitor. HBs were cultured in DPCGM containing 0, 10 or 50 μM LY294002 and incubated in non-cell-adhesive dishes for 72 hours. Changes of the HBs were observed by a phase-contrast microscopy after 0, 3 and 72 hours of culture. The expression levels of both trichogenic and PI3K/Akt signaling-related genes were assessed using RT-PCR. The methods of RNA extraction, reverse transcription, and RT-PCR were described above in 2.2.4. Primer sequences were also the same as in Table 1. Hair generation efficiency was quantified using a patch assay.

2.2.7. The hair generation efficiency by patch assay

The animal study was approved by the Committee on Animal Care and Use, Yokohama National University (Permit Number: 2019-04). All mouse care and handling procedures were conducted in strict accordance with the requirements of the Committee on Animal Care and Use at Yokohama National University. Embryonic mice (E18) were removed from C57BL/6 pregnant mice (CLEA, Tokyo, Japan) under sterile conditions. The epidermis from the outer edge of the ear to the buttocks was cut, and the dorsal skin areas were extracted. After incubating the dorsal skin with 4.8 U/mL sterilized dispase II (Roche Diagnostics GmbH; Mannheim, Germany) in a 4 °C refrigerator for 60 minutes, the epithelial and mesenchymal layers of the skin were separated using two tweezers. The separated epithelial and mesenchymal layers were incubated with 100 U/mL type I collagenase (Fujifilm; Osaka, Japan) for 80 minutes at 37 °C. The epithelial layers were treated with 0.25% trypsin for 10 minutes at 37 °C. After stopping the action of the enzyme, the cells were detached to the maximum extent by gentle pipetting, and then dispersed tissues and blown cells were filtered through a 40 μm filter to obtain a relatively pure cell suspension. After centrifugation for 3 minutes at 1,000 rpm, epithelial were

resuspended in epidermal keratinocyte growth medium-2 (KG2; Kurabo, Japan).

HBs were exposed to different concentration of LY294002 for 3 days of culture. HBs (25) and freshly isolated mouse embryonic epithelial cells (2.5×10^5 cells) were cultured in DPCGM and KG2 at a 1:1 ratio and seeded in 96-well-round-bottom plates (PrimeSurface 96U; Sumitomo Bakelite Co., Ltd, Japan) for 1 day to form cell aggregates.

BALB/c nude mice (5–6 weeks old) were purchased from CLEA Japan. Under anesthesia, a syringe was used to create a space as deep as adipose tissue in the mouse's skin, called a patch. The cell aggregates were transplanted into patches of dorsal skin. The transplanted sites were observed by a digital camera (Tough TG-6; Olympus, Japan) after 3 weeks. A small piece of skin at the transplanted site was treated with 100 U/mL collagenase at 37 °C to isolate the generated hair shaft. The number of hair shafts was counted.

2.2.8. Activation of PI3K/Akt signaling pathway

SC79 (Abcam) is a PI3K/Akt activator. DPCs (5×10^4 cells/well) were cultured in a 6-well plate in DPCGM for 24 hours. DPCs were treated with 0 or 10 μM SC79 for up to 24 hours. Gene expression was assessed by RT-PCR. The methods of RNA extraction, reverse transcription, and RT-PCR were described above in 2.2.4. Primer sequences were also the same as in Table 1.

2.2.9. Statistical analysis

The data were expressed as mean value ± SE. SPSS statistics version 22 was used for statistical analysis. For pairwise comparisons, after ensuring the sample variance was equal, Student’s t-test was used to determine the statistical significance of differences between experimental and control groups. When the variable is more than two groups,

one-way ANOVA was conducted. Firstly, a test for homogeneity of variances was performed. According to the results of the F test, when $P > 0.05$, indicating that the sample variance was equal, one-way ANOVA with the Turkey test was used. According to the results of the F test, when $P \leqslant 0.05$, indicating that the sample variance was unequal, one-way ANOVA with Tamhane's T2(M) was used. The α level was 0.05. $p > 0.05$ indicated that the difference was not statistically significant and $p \leqslant 0.05$ indicated that the difference was statistically significant. When $P \leqslant 0.05$, "*" was expressed in the following charts.

2.3. Results and discussion

2.3.1. Gene expression analysis

In this study, transcriptome analysis of human DPCs in 2D and HB cultures was performed to identify the predominant intracellular signaling pathways responsible for hair induction. Firstly, we confirmed whether the trichogenic genes were upregulated after 3 days of HB culture compared to those before cultivation and after 3 days of 2D culture (Figure 9A). The expression of typical trichogenic genes of DPCs, including Versican, ALP, LEF1 and Wnt5a [72], was significantly upregulated in HBs, but downregulated in 2D culture compared to before cultivation (Figure 9B).

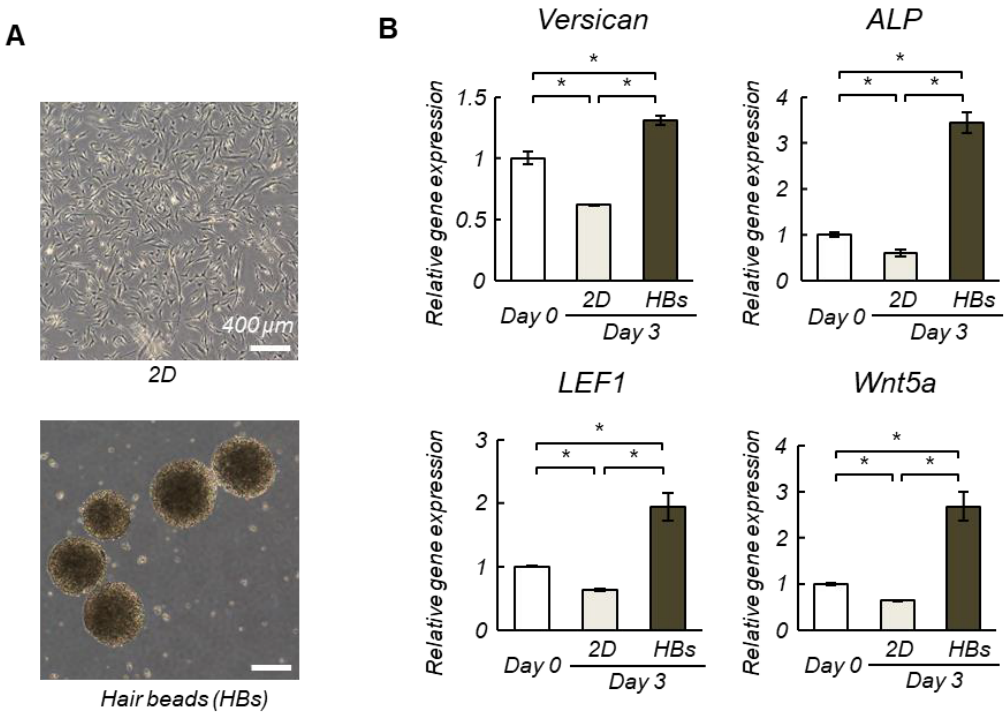

Figure 9. Gene expression analysis of DPCs cultured in 2D and HB cultures [71]. (A) Phase-contrast microscopic images of DPCs in 2D and HB cultures at 3 days of culture. (B) Expression of trichogenic genes.

2.3.2. Gene chip analysis

The gene chip assay revealed a total of 1924 differentially expressed genes (DEGs) between 2D and HB cultures, and these were used for pathway analysis. The top 10 pathways included those associated with hair follicle morphogenesis, such as the PI3K/Akt signaling pathway, the mitogen-activated protein kinase (MAPK) signaling pathway, and the transforming growth factor-β (TGFβ) signaling pathway (Figure 10A). The human papillomavirus infection topped the list, however, many of the genes listed in this group are common in the PI3K/Akt signaling pathway. They are also frequent in focal adhesions and apoptosis. Thus, the human papillomavirus infection and focal adhesion have no special research value.

The PI3K/Akt signaling pathway was thought to be necessary in hair follicle regeneration in mice [73]. In drug treatment, finasteride can improve the stemness of

human DPCs by activating Akt protein [74]. TGFβ2 promotes collagen secretion and enhances human DPC maintenance and hair induction [75]. Upregulation of these pathways may explain the enhanced hair-inducing ability in HB culture. Since the PI3K/Akt signaling pathway ranked second among the top 10 enriched pathways, the expression of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) and AKT serine/threonine kinase 1 (AKT1) were examined in this chapter by RT-PCR. PIK3CA is a catalytic subunit of PI3K, and AKT1 is a downstream gene of PI3K [76]. The expression of these two genes was significantly up-regulated in HBs (Figure 10B) , which was as expected.

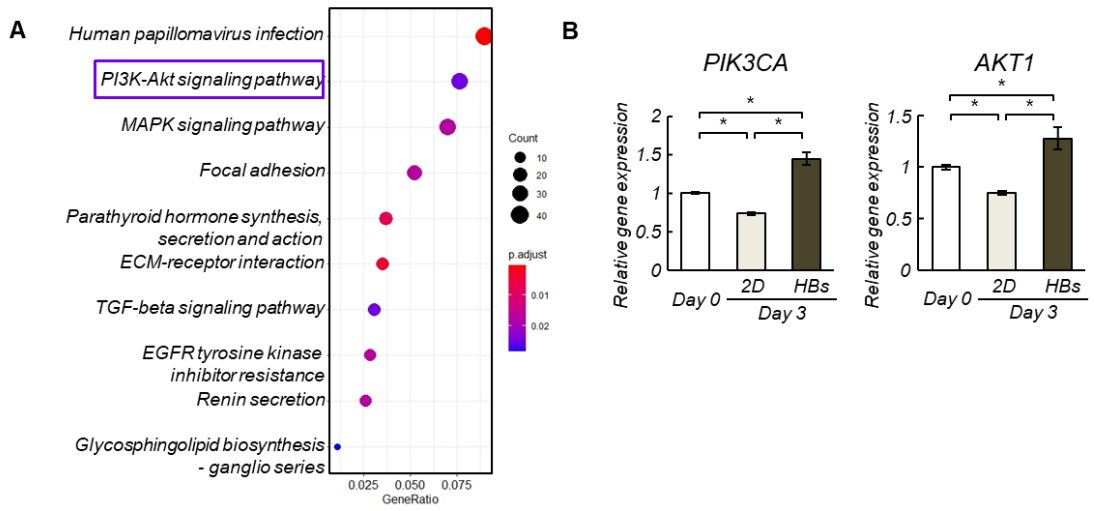

Figure 10. Transcriptome comparison [71]. (A) KEGG analysis. The top 10 enriched pathways were identified with differentially expressed genes (DEGs). (B) Gene expression related to the PI3K/Akt signaling pathway. *, P≤0.05.

2.3.3. Inhibition of PI3K/Akt signaling pathway

According to the results from the study by Kageyama et al. [70], HBs contracted spontaneously by cellular attraction. But the contraction was delayed with the supplementation of LY294002 (Figure 11A). This delay caused by LY294002, a PI3K/Akt inhibitor, may be because PI3K/Akt signaling is closely related to integrin-mediated cell

adhesion. The activation of PI3K/Akt signaling has been reported to promote integrin-mediated adhesion of human dermal fibroblasts to collagen type I, while the PI3K/Akt inhibitor is able to inhibit it [77].

The expression levels of both trichogenic genes (including Versican, ALP, LEF1 and Wnt5a) and PI3K/Akt signaling-related genes (including PIK3CA and AKT1) were significantly downregulated by the supplementation with LY294002 (Figure 11B and C). It shows that the PI3K/Akt signaling pathway is closely related to the hair-forming function of DPCs.

The animal experiment involving PI3K/Akt inhibitor was performed. HBs were exposed to 0, 10 or 50 μM LY294002 for 3 days and mixed with freshly isolated mouse embryonic epithelial cells to form cell aggregates. The cell aggregates were transplanted into patches of dorsal skin of nude mice. After 3 weeks, clumps of black hair shafts were observed (Figure 11D). The number of generated hairs was counted in Figure 11E, showing that inhibition of the PI3K/Akt pathway significantly reduced hair neogenesis. These results suggest that the PI3K/Akt signaling pathway is critical for hair follicle generation by DPCs.

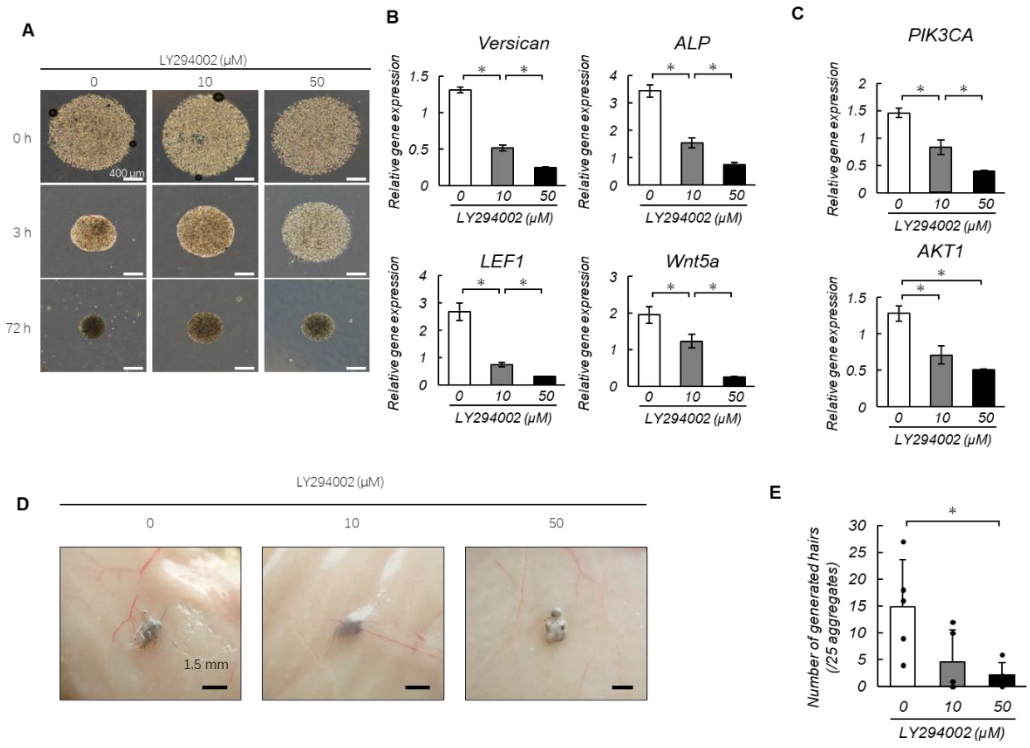

Figure 11. Inhibition of PI3K/Akt signaling pathway [71]. (A) Phase contrast microscopy images of HBs incubated with the PI3K/Akt inhibitor of LY294002 for 0, 3 and 72 hours. (B) Expression of trichogenic genes of DPCs. (C) Expression of PI3K/Akt signaling-related genes. (D) The hair generation efficiency by patch assay after 3 weeks of transplantation. (E) The number of generated hairs.

2.3.4. Activation of PI3K/Akt signaling pathway

The effects of SC79, an activator of the PI3K/Akt signaling pathway, on the gene expression of DPCs in 2D culture were discussed. The trichogenic and PI3K/Akt signaling-related genes were significantly upregulated by the supplementation with SC79 (Figure 12A, B). These results suggest that an activator of the PI3K/Akt signaling pathway can be used to increase the hair-inducing ability of DPCs in culture.

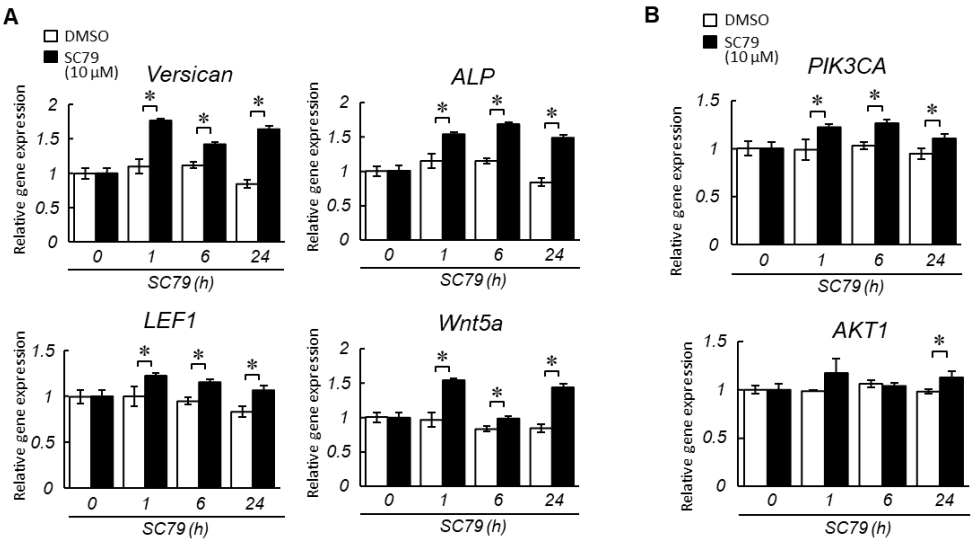

Figure 12. Activation of PI3K/Akt signaling pathway on DPCs in 2D culture [71].

2.4. Conclusion of this chapter

In summary, the trichogenic gene expression was significantly upregulated in HB culture, but downregulated in 2D culture in this chapter. Transcriptome comparison showed that PI3K/Akt signaling pathway plays an important role in promoting hair growth. Inhibition of the PI3K/Akt signaling pathway decreased the hair-inducing

capability of DPCs, while the activation of the PI3K/Akt signaling pathway improved gene expression of DPCs. It suggests that the PI3K/Akt is the underlying intracellular signaling pathways of DPCs responsible for hair induction, which is instructive for remodeling and maintaining DPC function *in vitro*.

CHAPTER 3

3. Extracellular signaling between dermal papilla cells and hair follicle epithelial cells

3.1. Purpose

This chapter aimed to explore extracellular signaling of DPCs responsible for hair induction and to understand communication between epithelial and dermal cells via exosomes. Another intention was to find out the differences in exosomes obtained by different culture methods of DPCs in order to develop new directions for the treatment of hair loss.

3.2. Materials and Methods

3.2.1. Overview of experimental steps

Human DPCs were cultured using typical two-dimensional culture (2D), spheroid culture (3D), and spheroid microwell array culture in an oxygen-permeable substrate (3D-oxy). Exosomes secreted into the media during these culture methods were collected and purified. The size and shape of the prepared exosomes, as well as the contained surface proteins, were then examined. Purified exosomes were exposed to human DPCs, hair follicle epithelial cells, and hair follicle organoids composed of mouse embryonic epithelial and mesenchymal cells (Figure 13).

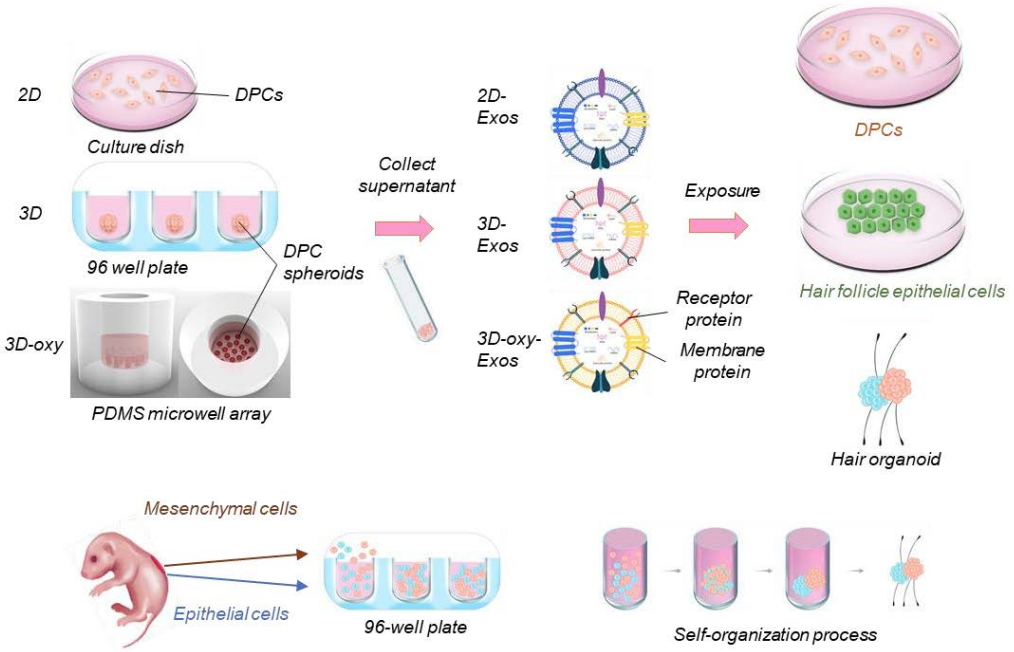

Figure 13. A Schematics of the experimental procedure and steps in this chapter

3.2.2. Culture of dermal papilla cells

Human DPCs at passage 2 (PromoCell GmbH, Heidelberg, Germany) were grown in the culture medium of DPCGM (Follicle Dermal Papilla Cell Basal Medium and Follicle Dermal Papilla Cell Growth Medium Supplement Pack; PromoCell GmbH, Heidelberg, Germany) and incubated at 37 °C in 5% CO_2 (CO_2 incubator; PHCbi, Japan). After the cells were approximately 80% adherent, they were passaged by trypsin treatment (0.25% Trypsin-EDTA; Gibco, NY, USA). After the cells adhered to about 80% again, they were treated by cell banker (CELLBANKER 1; ZENOGEN PHARMA, FKS, Japan) and stored in liquid nitrogen for cryopreservation.

Human DPCs at passage 4 were washed thrice with phosphate-buffered saline (PBS), then suspended in the exosome-depleted culture media (see below), seeded in a conventional two-dimensional (2D) and three-dimensional (3D) culture and lab-made spheroid microwell array fabricated with oxygen-permeable polydimethylsiloxane (PDMS) (3D-oxy) (Figure 13). The exosome-depleted culture medium included 500 mL

high glucose Dulbecco′s modified Eagle′s Medium (DMEM; Sigma-Aldrich, Japan G.K.), 50 mL Exo-FBS (Exosome-depleted FBS; System Biosciences, CA, USA), 5 mL PenStrep stock (10,000 U/mL Pen, 10 mg/mL Strep, Sigma-Aldrich Japan G.K.), 0.5 μg basic fibroblast growth factor and 2.5 mg insulin (from Follicle Dermal Papilla Cell Growth Medium Supplement Pack, PromoCell GmbH, Heidelberg, Germany). In the 2D culture, DPCs were seeded at 2×10^5 cells/10 mL in a 10 cm dish (TPP, Japan). In the 3D culture, a total of 2×10^5 cells were seeded in a 96-well-round-bottom plate (PrimeSurface 96U; Sumitomo Bakelite Co., Ltd, Japan) with 5000 cells/200 μL/well. In the 3D-oxy culture, a total of 2×10^5 cells were seeded in a PDMS plate, which was fabricated as previously reported [78], with 5000 cells/microwell. Briefly, the prepolymer solution and curing agent of PDMS (SYLGARD 184 Silicone Elastomer Kit, Toray Daw Corning, Japan) were mixed at a ratio of 10:1 and then placed on a mold at 100 °C for 1 hour. After oxygen etching, the plates were coated with Prevelex (Nissan Chemical Co., Japan) for anti-cell adhesion. Then 25 kGy of gamma irradiation was used for sterilization. One PDMS plate had 19 microwells and required 200 μL of the culture media. Every 48 hours, the culture media were collected and temporarily stored in a -80 °C freezer.

3.2.3. Culture of hair follicle epithelial cells

Primary hair follicle epithelial cells were extracted from patients with AGA. All experimental procedures were performed in accordance with protocols approved by the Institutional Ethical Committee, YNU Ethical Committee for Medical and Health Research (Authorization No. Hitoi-2018-16), and following the ethical guidelines for medical and health research involving human subjects from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare, Japan. The patient provided informed consent to collect and use the hair follicles damaged during hair transplantation. One microliter per well of Easy iMatrix-511 (MATRIXOME; Osaka, Japan) was added to a 6-well plate, and incubated at 37 °C for at least 1 hour.

Under the low temperature created by ice packs, human hair follicles were transferred to a 6 cm dish containing DMEM-HEPES (Gibco; NY, USA) solution to remove visible hair debris. The dermal papilla was separated using specialized forceps and scissors (Figure 14). The upper tissue, including bulge parts was transferred to a 6 cm dish containing Dispase/Collagenase solution (4.8 U/mL of dispase II and 100 U/mL of collagenase in a 1:1 mixture of PBS and HBSS) (Roche Diagnostics GmbH, Mannheim, Germany). The bulge tissue was incubated at 37 °C for 10 minutes. Then 5 mL of DMEM-HEPES solution was used to stop enzymatic reaction. Tweezers were used to peel and dispose of the visible collagen sheath around the hair shaft. The bulge tissue stripped from the sheath was transferred to a new 6 cm dish. The bulge tissue was placed in a new 15 mL tube containing 5 mL of 0.05 v/v% trypsin solution (Trypsin-EDTA; Gibco, NY, USA), followed by incubation at 37 °C for 45 minutes. Next, a 40 μm cell strainer (Corning Incorporated; NY, USA) was used for sterilization and DMEM+ solution (DMEM+10% FBS+1% PenStrep stock) was used to stop the enzymatic reaction. The Easy iMatrix liquid was removed from the 6-well plate to which hair follicle epithelial cells suspended in stemfit+Y+A medium (Stemfit+10 μM Y27632+5 μM A83-01; Ajinomoto, Tokyo, Japan) were seeded. Hair follicle epithelial cells at passage two were cryopreserved and stored in liquid nitrogen until further use. The prepared hair follicle epithelial cells contain HFSCs and other types of epithelial cells.

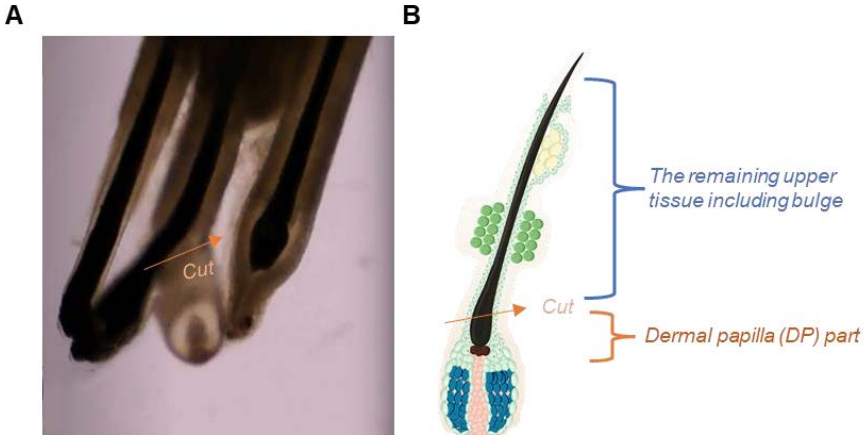

Figure 14. Separation of dermal papilla and upper tissue including bulge. (A) Screenshot of the experimental teaching video shot by Dr. Tatsuto Kageyama. (B) Schematic diagram.

3.2.4. Exosome isolation

3.2.4.1. Preparation of exosomes by ultracentrifugation

The method used in the study was referred to a study by Zhou et al. [67]. The culture media were centrifuged at 300×*g* for 10 minutes and then 1,500×*g* for 10 minutes. The culture media were passaged through 0.22-μm filters to ultra-15 centrifugal filter units with 100 kDa NMWCO (Amicon, Merck Millipore, MA, USA). The samples were centrifuged at 4,000×*g* in a swinging-bucket rotor for about 20 minutes until the volume of ultrafiltration liquid remaining in the upper compartment was about 500 μL. The ultrafiltration liquid was washed twice with PBS and centrifuged at 4,000 × *g* in a swinging-bucket rotor for about 20 minutes until the ultrafiltration liquid in the upper compartment was 500 μL again. For exosome purification, the samples were ultracentrifuged at 100,000×*g* for 2 hours at 4 °C (himac CP70MX; Eppendorf Himac Technologies, Japan). The pelleted exosomes were resuspended in PBS. The samples were passaged through 0.22-μm filters, followed by transferred to ultra-15 centrifugal filter units and centrifuged at 4,000×*g* by swinging-bucket rotor for about 25 minutes until the volume of ultrafiltration liquid remaining in the upper compartment was about 300 μL. Finally, exosomes were stored at a -80 ℃ freezer until further use (Figure 15).

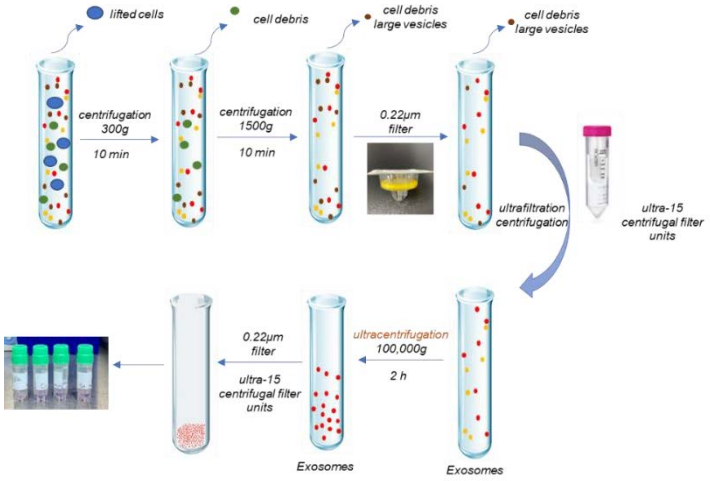

Figure 15. The steps of preparing exosomes by ultracentrifugation

3.2.4.2. Preparation of exosomes by polymer precipitation

There are commercial kits for the preparation of exosomes by polymer precipitation. In the present study, exosomes were isolated according to the manufacturer's protocol for the Total Exosome Isolation Kit (Invitrogen, Carlsbad, CA, USA). The culture media collected from the three culture methods (including 2D, 3D, and 3D-oxy cultures) were centrifuged at 2,000×*g* for 30 minutes to remove cells and debris. The total exosome isolation reagent was added and mixed thoroughly, followed by incubation at 4 °C overnight. After incubation, they were centrifuged at 10,000×*g* for 60 minutes at 4 °C. The exosome pellets were re-suspended in 1×PBS. Isolated exosomes were kept in a -80 °C freezer until further use (Figure 16).

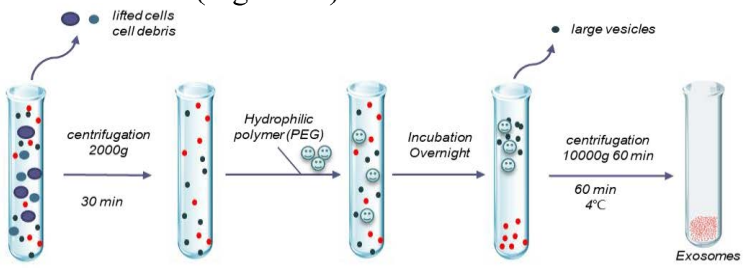

Figure 16. The steps of preparing exosomes by polymer precipitation method

3.2.5. Concentration of exosomal protein and exosome quantitation

BCA protein assay (Pierce, Thermo Fisher Scientific, WI, USA) was performed according to the manufacturer's protocol. Briefly, serial dilution was used to prepare diluted albumin (BSA) standards and the BCA working reagent (WR) was made at room temperature (25 °C). A total of 25 µL of standard samples or exosome samples were added to each well of a 96-flat-well plate. WR (200 µL) was added to each well and mix thoroughly, followed by incubation at 37 °C for 30 minutes. The plate was read at 562 nm by EnSpire Multimode Plate Reader. The corrected absorbance was obtained by subtracting the absorbance of the blank (without exosomes) from the initial absorbance measured at 562 nm. The corrected absorbance of different concentrations of BSA standard was prepared to a standard curve. The abscissa was the concentration, in µg/mL, and the ordinate was the corrected absorbance at 562 nm. The standard curve was used to determine the protein concentration of each exosome sample.

Exosome concentration was conducted according to the manufacturer's protocol for the EXOCET Exosome Quantitation Kit (System Biosciences, CA, USA). Briefly, 20–100 µg exosome protein quantified using the BCA assay was used in every reaction. Exosomal protein samples were prepared using lysis buffer and centrifugation. The EXOCET standard curve was prepared using serial dilution. Fresh EXOCET reaction buffer (50 µL) was added to a 96-well plate, followed by addition of 50 µL of standard solution or exosome samples. The plate was incubated for 20 minutes at room temperature. The plate was read at 405 nm using an EnSpire multimode plate reader.

3.2.6. Scanning electron microscopy (SEM)

The morphology of exosomes was investigated using SEM according to a previous

report [79]. The exosome pellets were fixed in PBS containing 2.5% glutaraldehyde for 1 hour at room temperature. The samples were transferred to an Ultra-clear tube (Amicon Ultra-15 Centrifugal Filter Unit; 10 kDa, Merck KGaA, Darmstadt, Germany) and washed by adding 10 mL PBS. The samples were centrifuged at 4,000×*g* using a swinging-bucket rotor for 15 minutes until the ultrafiltration liquid in the upper compartment was approximately 200 μL. The exosome pellets were resuspended in 100 μL of PBS and placed on fibronectin-precoated 22 mm round coverslips (Corning BioCoat Fibronectin 22 mm Round, Corning Life Sciences, MA, USA); the moisture on the coverslips was maintained. The samples were incubated overnight at 37 °C. On the second day, the coverslips were dehydrated in ethanol concentrations from 30%, 50%, 70%, 80%, 90% to 95% for 5 minutes per concentration, followed by two 5 minutes incubations in 100% ethanol. The samples were then chemically dried in 100% hexamethyldisilazane (Sigma-Aldrich, Japan G.K.) for 3 minutes and allowed to air dry at 37 °C for approximately 1 hour. The samples on the coverslips were coated with gold particles (Magnetron Sputter; MSP-30T, IBS, Japan). The exosomes were observed under a scanning electron microscope (Hitachi SU8010).

3.2.7. Exosome Antibody Arrays

Exosome purity was examined according to the manufacturer's protocol for the Exo-Check Exosome Antibody Arrays (System Biosciences, CA, USA). The 10× lysis buffer was mixed with 50 μg exosome samples to a final concentration of 1×. One microliter of labeling reagent was added, and the samples were incubated at room temperature (25 °C) for 30 minutes. A column buffer was used, and the samples were purified by centrifuging the columns several times. Labeled exosome lysates (50 μg) were added to 5 mL blocking buffer. The labeled exosome lysate with blocking buffer mixture (5 mL) was dropped on a membrane and incubated at 4 °C overnight with constant gentle shaking. One type of exosome was used on each membrane. On the second day, the membrane was washed

several times with wash buffer. Detection buffer (5 mL) was added to the membrane and incubated for 30 minutes at room temperature. The detection buffer was removed, and the membrane was washed several times. The developer mixture (1.5 mL; Amersham ECL Prime western blotting detection reagents; GE Healthcare UK Limited, Bucks, UK) was incubated on the membrane for 5 minutes. The membranes were imaged using a CCD camera system (Amersham Imager 600).

3.2.8. Proliferation of human dermal papilla cells

The proliferation of DPCs was measured using the CCK8 assay (Abcam, CBG,UK). DPCs (5000 cells/100 μL/well) were cultured in exosome-depleted culture media, seeded in 96-flat-well plates and placed in the incubator for pre-culture for 24 hours. Then, 2D-Exos (100×10^7 particles/10 μL/well), 3D-Exos (100×10^7 particles/10 μL/well) and 3D-oxy-Exos (100×10^7 particles/10 μL/well) were then added to each well. PBS (10 μL/well) was used as a control (No Exo). After 24h, 48h, and 72h of incubation, 10 μL/well of CCK8 solution was added to each well. The 96-well plate was incubated for 30 minutes at 37 °C in the dark. The absorbance of the plate was measured at 460 nm using an EnSpire multimode plate reader.

3.2.9. Proliferation of human hair follicle epithelial cells

The proliferation of hair follicle epithelial cells was measured using the CCK8 assay. Cells (5000 cells/100 μL/well) were cultured in Stemfit+Y+A, seeded in 96-flat-well plates and placed in the incubator for pre-culture for 24 hours. Then, 2D-Exos (210×10^7/10 μL/well), 3D-Exos (210×10^7/10 μL/well) and 3D-oxy-Exos (210×10^7/10 μL/well) were then added to each well. PBS (10 μL/well) was used as a control (No Exo). After 24h, 48h, and 72h of incubation, 10 μL/well of CCK8 solution was added to each well. The 96-well plate was incubated for 30 minutes at 37 °C in the dark. The absorbance

of the plate was measured at 460 nm using an EnSpire multimode plate reader.

3.2.10. Preparation of hair follicle organoids

Embryonic mice (E18) were removed from C57BL/6 pregnant mice (CLEA, Tokyo, Japan) under sterile conditions. The epidermis from the outer edge of the ear to the buttocks was cut, and the dorsal skin areas were extracted. After incubating the dorsal skin with 4.8 U/mL sterilized dispase II (Roche Diagnostics GmbH; Mannheim, Germany) in a 4 °C refrigerator for 60 minutes, the epithelial and mesenchymal layers of the skin were separated using two tweezers. The separated epithelial and mesenchymal layers were incubated with 100 U/mL type I collagenase (Fujifilm; Osaka, Japan) for 80 minutes at 37 °C. The epithelial layers were treated with 0.25% trypsin for 10 minutes at 37 °C. After stopping the action of the enzyme, the cells were detached to the maximum extent by gentle pipetting, and then dispersed tissues and blown cells were filtered through a 40 μm filter to obtain a relatively pure cell suspension. After centrifugation for 3 minutes at 1,000 rpm, epithelial and mesenchymal cells were resuspended in Advanced DMEM/F12 (Gibco; NY, USA). The epithelial and mesenchymal cells were then mixed 1:1 and seeded in a 96-round-bottom well plate (PrimeSurface 96U; Sumitomo Bakelite Co., Ltd, Japan) at a concentration of 1×10^4 cells/150 μL/well for each type of cells. 2D-Exos, 3D-Exos and 3D-oxy-Exos were added to the wells at a concentration of 30×10^7/20 μL/well, while control wells used the same volume of PBS.

3.2.11. Gene expression analysis

Human DPCs (5×10^4 cells/2 mL/well) were cultured in exosome-depleted culture media, seeded in a 6-well plate and put in the incubator for pre-culture for about 24 hours. After cells' adhesion, culture media were changed, and then 2D-Exos (80×10^7/67

μL/well), 3D-Exos(80×10^7/67 μL/well) and 3D-oxy-Exos (80×10^7/67 μL/well) were supplemented into each well respectively. PBS (67 μL/well) was set as control reference. 24 hours after exosomes treatment, the cells were washed thrice with PBS and total RNA was extracted.

Human hair follicle epithelial cells (5000 cells/100 μL/well) were suspended in Stemfit+Y+A, seeded in 96-flat-well plates, and pre-incubated for 24 hours. 2D-Exos (210×10^7/10 μL/well), 3D-Exos (210×10^7/10 μL/well), 3D-oxy-Exos (210×10^7/10 μL/well) were supplemented into each well. After 24, 48, and 72 hours of culture, the cells were washed thrice with PBS and total RNA was extracted.

Mouse hair follicle organoids were exposed to exosomes from the three different culture methods for 3 days. The hair follicle organoids were washed thrice with PBS and homogenized.

Total RNA was then extracted using a RNeasy Mini Kit (250) (QIAGEN, Hilden, Germany) and QIAshredder (250) (QIAGEN, Hilden, Germany). All RNA were dissolved in nuclease-free Water. RNA concentration was measured using NanoDrop One (Thermo Fisher Scientific; WI, USA). Nuclease-free water was used to dilute all RNA samples to equal concentration. Reverse transcription was conducted using the ReverTrA ace qPCR RT kit (Toyobo, Osaka, Japan). cDNA was obtained by incubating the samples at 37 °C for 15 minutes and then 95 °C for 5 minutes in QuantStudio 3 (Applied Biosystems by Thermo Fisher Scientific). The cDNA samples were stored at -30 °C until RT-PCR was performed. RT-PCR was conducted with TB Green Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio, Shiga, Japan) using the corresponding forward and reverse primers (synthesized by FASMAC,Kanagawa, Japan), listed in Tables 2, 3, and 4. Amplification was performed under the following cycling conditions: 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. The experiments were conducted in triplicate at each time point for each culture method. The $2^{-\triangle\triangle Ct}$ method was used to compare the relative mRNA expression levels of the target

genes to GAPDH expression.

Gene name	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
Versican	CCAGCAAGCACAAAATTTCA	TGCACTGGATCTGTTTCTTCA
ALP	ATTGACCACGGGCACCAT	CTCCACCGCCTCATGCA
LEF1	CTTCCTTGGTGAACGAGTCTG	TCTGGATGCTTTCCGTCAT
BMP4	GCCCGCAGCCTAGCAA	CGGTAAAGATCCCGCATGTAG
NOG	CTGGTGGACCTCATCGAACA	CGTCTCGTTCAGATCCTTTTCCT

Table 2. The primer sequences for the RT-PCR analysis of human DPCs

Gene name	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	TGGAAATCCCATCACCATCTTC	CGCCCCACTTGATTTTGG
CD200	TGACTCTGTCTCACCCAAATG	GCTTAGCAATAGCGGAACTG
CD34	CTACAACACCTAGTACCCTTGGA	GGTGAACACTGTGCTGATTACA
K15	GACGGAGATCACAGACCTGAG	CTCCAGCCGTGTCTTTATGTC
K14	GGCCTGCTGAGATCAAAGACTAC	CACTGTGGCTGTGAGAATCTTGTT
ITGA6	GCTGGTTATAATCCTTCAATATCAATTGT	TTGGGCTCAGAACCTTGGTTT

Table 3. The primer sequences for the RT-PCR analysis of human hair follicle epithelial cells

Gene name	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	AGAACATCATCCCTGCATCC	TCCACCACCCTGTTGCTGTA
Versican	GACGACTGTCTTGGTGG	ATATCCAAACAAGCCTG
IGFbp5	ATGAGACAGGAATCCGAACA	TCAACGTTACTGCTGTCGAA
TGFβ2	TCCCGAATAAAAGCGAAGAG	AAGCTTCGGGATTTATGGTG
Wnt10b	CCAAGAGCCGGGCCCGAGTGA	AAGGGCGGAGGCCGAGACCG

Table 4. The primer sequences for the RT-PCR analysis of mouse hair follicle organoids

3.2.12. Statistical analysis

The data of exosomal protein concentration, exosome concentration, exosome secretion rate, absorbance after exosome or control treatment and gene expression were

expressed as mean value ± SE. SPSS statistics version 22 was used for statistical analysis. Because the comparison of four groups of data (2D-Exos, 3D-Exos , 3D-oxy-Exos and controls) was involved, analysis of ANOVA was performed. There was one variable between multiple sets of data, that was, the effect of different kinds of exosomes or PBS on cells or tissues, one-way ANOVA was conducted. Firstly, a test for homogeneity of variances was performed. According to the results of the F test, when $P > 0.05$, indicating that the sample variance was equal, one-way ANOVA with the Turkey test was used. According to the results of the F test, when $P \leqslant 0.05$, indicating that the sample variance was unequal, one-way ANOVA with Tamhane's T2(M) was used. The α level was 0.05. $p > 0.05$ indicated that the difference was not statistically significant and $p \leqslant 0.05$ indicated that the difference was statistically significant. When $P \leqslant 0.05$, "*" was expressed in the following charts; when $P \leqslant 0.01$, "**" was expressed in the following charts.

3.3. Results and discussion

3.3.1. Literature support and cell sources

In the current research on exosomes in the hair field, in addition to the most widely used DPCs, mesenchymal stem cells (MSCs) are also mentioned. The related literature on MSCs-derived exosomes also has certain reference value. DPCs are mesenchymal cells with adult stem cell properties, whereas MSCs are adult stem cells that were originally identified in the bone marrow [80]. Adipose-derived stem cells (ADSCs) are a kind of MSCs that are obtained from adipose tissue [81]. The study by Zhang et al. [82] investigated the role of human umbilical cord MSC-derived exosomes (hucMSC-Ex) in skin wound healing. It found that HucMSC-Ex contained Wnt4, and activation of Wnt/β-catenin by hucMSC-Ex plays a critical role in skin injury healing. As mentioned above,

Wnt/β-catenin is also essential for hair growth. Another study by Rajendran et al. [83] cultured mouse bone marrow MSCs and collected the supernatant to prepare extracellular vesicles (MSC-EVs). It found that treatment with MSC-EVs resulted in increased proliferation and migration of DPCs, as well as increased levels of Bcl-2, phosphorylated Akt, and ERK. Furthermore, the expression and secretion of VEGF and IGF-1 in DPCs were increased with MSC-EVs. Intradermal injection of MSC-EVs into mice resulted in earlier transformation of telogen into anagen of hair growth cycle and increased expression of Wnt3a, Wnt5a and Versican. In 2021, Wu et al. [84] studied the effect of ADSCs-derived exosomes on hair regeneration. ADSCs were isolated from mice, and then ADSC-Exos were isolated from ADSCs. In the ADSC-Exos group, dermal cells, epidermal cells and exosomes were transplanted together into nude mice. In the control group, only dermal cells and epidermal cells were transplanted. It was found that two or three weeks after transplantation, the ADSC-Exos group had a higher number of regenerated hairs than the control group. Histologically, the ADSC-Exos group had more pronounced terminal hair and more follicles than the control group. This study focused on DPCs as a source of exosome-producing cells and was devoted to investigating communications between epithelial–dermal cells via exosomes (Figure 17).

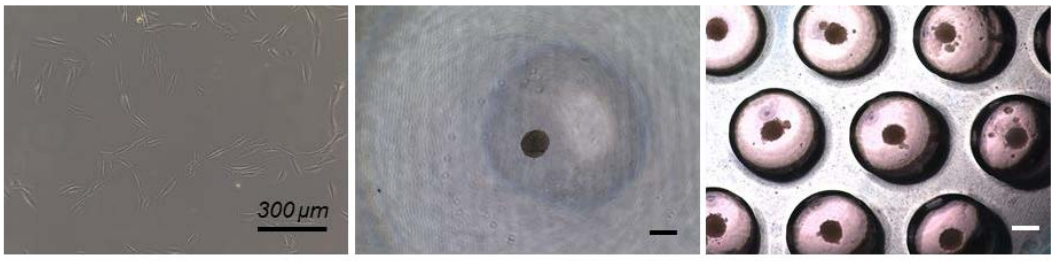

Figure 17. DPCs in the three different culture methods at 48 hours of culture. The cells show extended morphologies in 2D culture and spherical multicellular aggregates in 3D and 3D-oxy cultures.

3.3.2. Concentration of exosomal protein

The corrected absorbance of the sample was obtained by subtracting the initially

measured absorbance of the sample from the blank control. The corrected sample absorbance was calculated and compared with the standard curve to obtain the final sample protein concentration (Figure 18A). From Figure 18B, when the number of cells seeded and the volume of culture medium collected were the same in both preparation methods, the concentration of exosomal protein made by ultracentrifugation was only 91 μg/mL, while the concentration of exosomal protein obtained by the polymer precipitation was about 448 μg/mL. The latter was about 5 times that of the former.

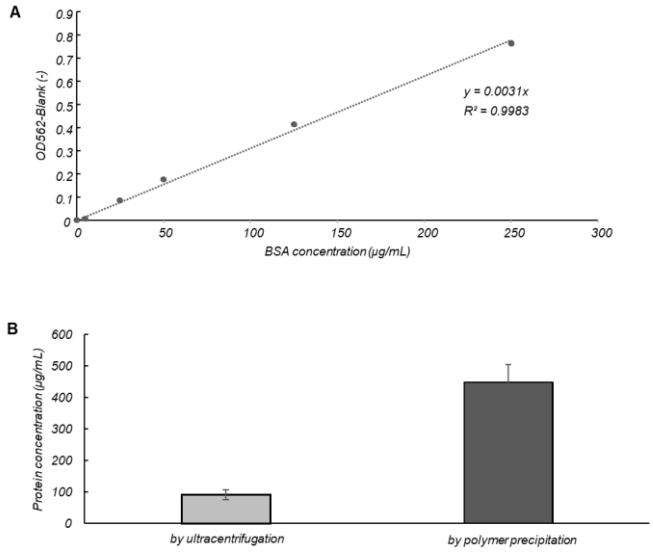

Figure 18. Protein concentration of exosomes. (A) Standard curve. (B) The result from two exosome preparation methods

3.3.3. Comparison among different exosome preparation methods

Regarding the isolation methods of exosomes, there are currently the following methods, including ultracentrifugation which can also be subdivided into different categories, as well as ultrafiltration, polymer precipitation, size-exclusion chromatography, immunoaffinity capture, microfluidics-based techniques and so on. Among them, some scholars believe that ultracentrifugation is the gold standard method for isolating exosomes [85]. According to an article, ultracentrifugation is the most widely

used method to isolate exosomes from the culture media of MSCs, accounting for about 54%, and the use of commercial kits ranks second, accounting for 27% [86]. Ultracentrifugation usually involves a speed of 100,000 to 150,000×*g* to prepare exosomes. Firstly, low-speed centrifugation such as 300×*g* is used to remove large unwanted particles, and then centrifugal force at different speeds from 1,500×*g* to 100,000×*g* is performed to sequentially remove contaminants, such as cell debris and apoptotic bodies. Finally, purified exosomes are obtained. Using this method, only an ultracentrifuge is required, and in theory, exosomes can be prepared indefinitely [85]. The ultrafiltration method uses nanomembranes with different molecular weight cut-off (MWCO), supplemented by centrifugation, to separate the required exosomes and other unwanted particles in samples [87]. One advantage of the ultrafiltration method is that it does not require the support of an expensive ultracentrifuge. However, one limitation of ultrafiltration method is that it is difficult to remove particles similar in size to exosomes. In addition, if the transmembrane pressure applied during the ultrafiltration process is not appropriate, it may have an adverse effect on exosomes [88]. The principle of polymer precipitation method is that the highly hydrophilic polymer is able to tie up water molecules surrounding the exosomes, forming a relatively hydrophobic microenvironment, leading exosomes to be precipitated [89]. Polyethylene glycol (PEG), as a kind of hydrophilic polymer, is a suitable non-toxic material. Firstly, samples need to be centrifuged to remove large cell debris, and then the sample supernatant and the PEG-containing solution are incubated together at 2–8 °C overnight. On the second day, the exosomal particles, which are not normally visible, are collected by centrifugation. This preparation method is simple to operate and requires a relatively low speed of the centrifuge, so it has been widely used to prepare exosomes from different types of samples, such as culture media of cells, serum, plasma, cerebrospinal fluid, urine, etc. [89].

From Figure 18B, the concentrations of exosomal proteins obtained by the two preparation methods were very different. Under the condition that the number of cells seeded and the volume of collected culture media were the same, the protein

concentration of exosomes prepared by the polymer precipitation was 5 times that of the ultracentrifugation.

In addition, the experimental steps of ultracentrifugation were more complicated, and the required instruments were also very special. The preparation method in this study consisted of multiple ultrafiltration centrifugations and one ultracentrifugation for up to two hours. The ultracentrifuge was located in National Institute of Health Sciences (NIHS). During the ultracentrifugation step, samples were ultracentrifuged at 100,000×*g* for 2 hours. In this process, the vacuum operation of the machine was necessary. Since rotational speed of ultracentrifugation was very fast, careful handling was a must.

In contrast, the polymer precipitation method was relatively simple to operate, and the most important thing required was the kit solution. The rotational speed used in the most important centrifugation step in this method was 10,000×*g* for 60 minutes at 4 °C.

Therefore, in the following experiments, the preparation method of polymer precipitation was used to make exosomes.

3.3.4. Exosome morphology

The characteristics of exosomes usually involve their diameter, morphology and surface antibodies. The diameter and morphology of exosomes can be confirmed by the following methods, such as electron microscopy, nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS) [90]. The types of electron microscopes reported in the literature for assessing the morphology of exosomes are scanning electron microscope (SEM) and transmission electron microscope (TEM). Both of these use electron beams to produce high-resolution images of particles [91]. The difference between the two is that in SEM, the emitted electrons interact with particles in the sample and scatter. Scattered electrons are detected, resulting in an image of the particles; in TEM, electrons pass through the sample and then be detected, resulting in an image [91]. For exosomes, although the displayed morphology is slightly different, both SEM and TEM are

applicable. Of course, because the operating principle of the machine is different, the way of preparing the sample is different. In the present experiment, the SEM detection method was selected according to the limitations of the instrument.

SEM analysis revealed that after the purification processes, the samples contained spherical particles with diameters between 100 and 160 μm (Figure 19), which is consistent with previous reports [60]. There were no apparent differences in the morphology of exosomes among the different culture methods.

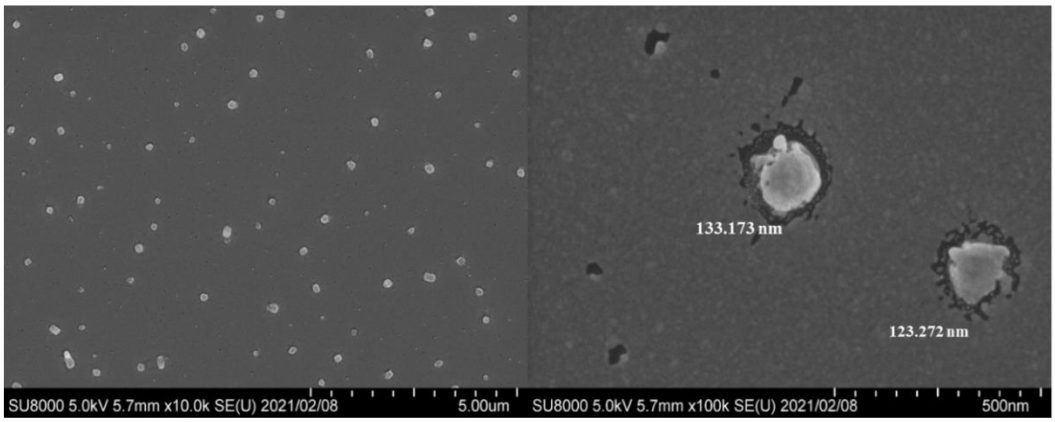

Figure 19. Scanning electron microscopy images of exosomes.

3.3.5. Exosome quantitation

As for the quantitation and concentration of exosomes, due to the limitations of the instrument, NTA was not used for detection, but a kit related to NTA was selected. The working principle of the kit is the presence of AChE in exosomes, and this enzyme can be directly detected at the absorbance of OD405. Standard is included in the kit to prepare a standard curve. Due to the relatively high requirements for storage conditions of exosomes, this standard does not contain exosomes, but the signals observed for a certain number of exosomes are calibrated according to NTA.

Figure 20 showed the exosome quantitation. A standard curve was prepared according to the instructions. The corrected sample absorbance was calculated and compared with the standard curve to obtain the final exosome concentration (Figure 20A).

From Figure 20B, in the case of the same number of cells, the 2D culture produced a higher concentration of exosomes than the 3D culture, and the 3D culture produced a higher concentration of exosomes than the 3D-oxy culture. To be precise, the concentration of prepared 2D-Exos was 286×10^9 particles/mL, and the concentration of 3D-Exos was 190×10^9 particles/mL. The concentration of 3D-oxy-Exos was 27×10^9 particles/mL.

Of course, in addition to the number of cells, the final amount of exosome was also related to the volume of culture medium in the following collecting steps. As a result, the exosome secretion rate was calculated by normalizing the average number of exosomes to the volume of the culture medium and the cell number in the culture methods (Figure 20C). The results indicated that there was no statistical difference among the three methods (2D-Exos vs. 3D-Exos: $p = 0.996$; 2D-Exos vs. 3D-oxy-Exos: $p = 0.363$; 3D-Exos vs. 3D-oxy-Exos: $p = 0.374$). The average secretion rate in 3D-oxy culture (3D-oxy-Exos) was almost twice that in the 2D (2D-Exos) and 3D cultures (3D-Exos).

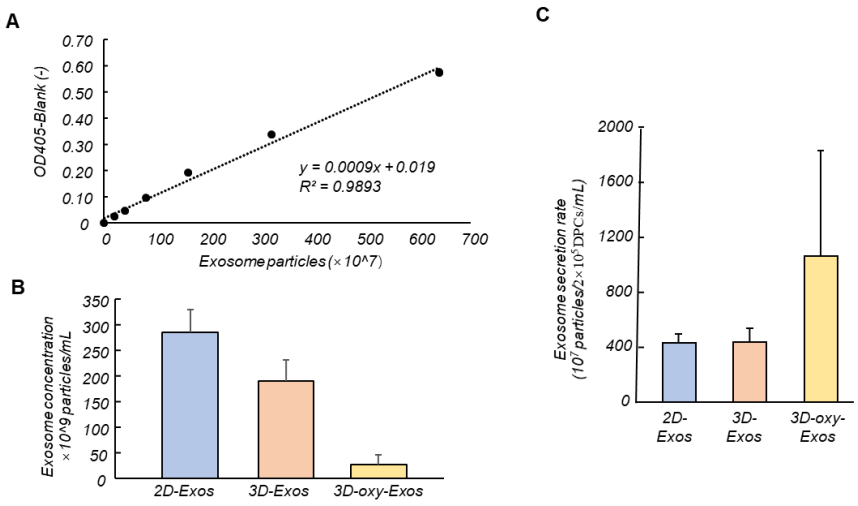

Figure 20. Exosome quantitation. (A) Standard curve. (B) The concentration of three types of exosomes. (C) Exosome secretion rate. The rate was calculated by normalizing the average number of exosomes secreted in 48 hours to the number of cells and the volume of culture medium.

3.3.6. Surface antibody

The surface antibodies can be determined by staining, immunoblotting or proteomics analysis [88]. The surface antibodies contained in exosomes are related to their formation process. Since the formation of multivesicular bodies (MVBs) and exosomes is regulated by the ESCRT protein, no matter which type of cells the exosomes are produced, theoretically exosomes should contain the ESCRT protein and its accessory proteins (such as Alix, TSG101, etc.) [92]. CD63, CD9, and CD81 are proteins in the tetraspanin protein family, which are usually found in exosomes [93]. In contrast, proteins related to the Golgi apparatus and endoplasmic reticulum should theoretically be negative in exosomes. But in fact, these proteins can still be detected at low concentrations, because early endosomes can interact with Golgi apparatus and endoplasmic reticulum [90].

In this study, the surface markers of exosomes (Figure 21), such as CD63, EpCAM, ANXA5, TSG101, FLOT1, ICAM1, ALIX, and CD81, were positive. In contrast, GM130 was negative, which localizes mainly to the Golgi and is a signal of cell contamination in the preparation of exosomes. These results showed that exosomes were prepared without cell contamination from the three culture methods

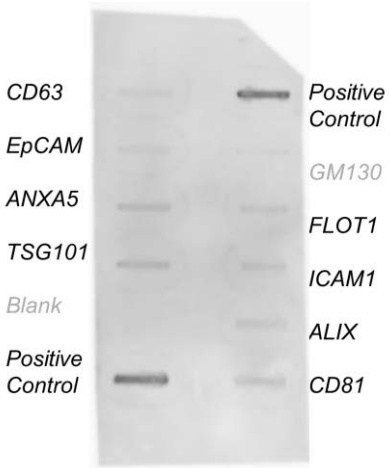

Figure 21. Detection of the surface markers for exosomes. The antibody array contains positive (black font) and negative (gray font) markers for exosomes.

3.3.7. The effect of exosomes on the proliferation of dermal papilla cells

I investigated the effects of exosomes derived from DPCs on the proliferation of human DPCs. “No Exo” was used as the label of PBS. “Preincubation” referred to photos taken under the microscope after the cells were pre-incubated for 24 hours and before the treatment of exosomes or the same volume of PBS. The same amounts of exosomes were added to the culture media, and DPCs were cultured in a conventional culture dish for 24, 48 to 72 hours. The cells partially covered the surface of culture dish and showed similar morphology (Figure 22).

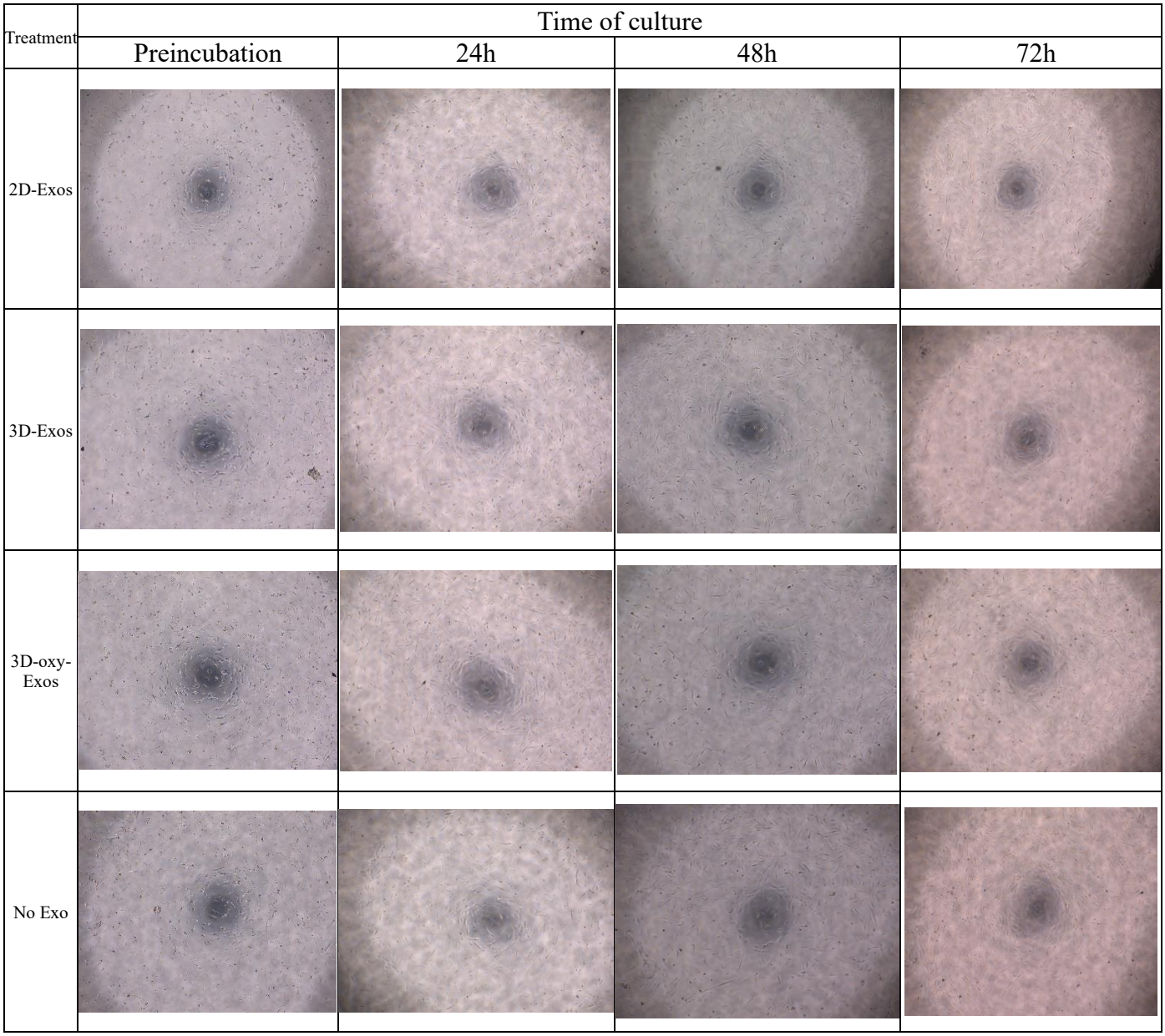

Figure 22. Phase-contrast microscopic images of DPCs (4×).

The effects of exosomes on the proliferation of human DPCs were analyzed by CCK8 assay (Figure 23). At 24 hours, there was no significant difference between exosomes from different sources and the control groups. At 48 hours, exosomes from 2D culture promoted the proliferation of DPCs and there was a trend of continuous promotion. At 72 hours, exosomes from various sources promoted the proliferation of DPCs, with 2D-Exos being the strongest, followed by 3D-Exos. However, based on statistical calculations, at 24 hours, 48 hours and 72 hours, there were no significant difference between 2D-Exos,

3D-Exos, 3D-oxy-Exos and controls ($p > 0.05$).

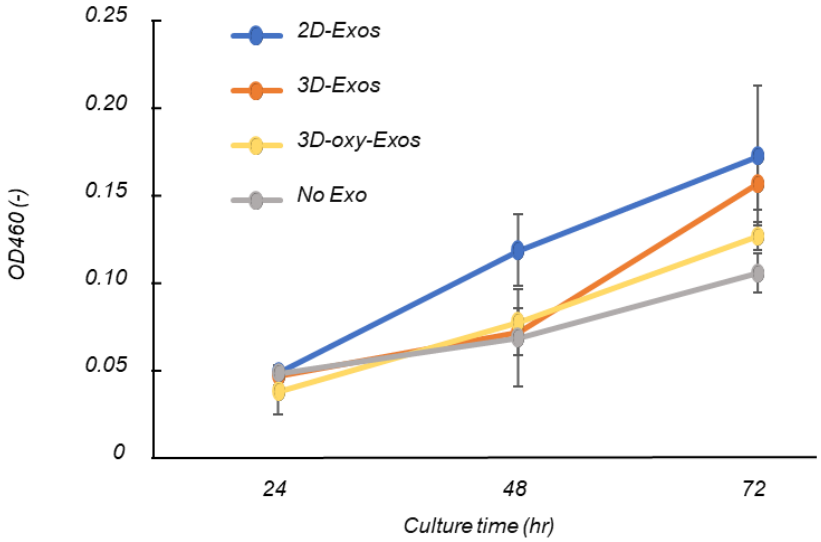

Figure 23. The effects of exosomes on the proliferation of human DPCs

3.3.8. The genetic impact of exosomes on dermal papilla cells

Maintenance of hair-inducing properties of DPCs was investigated using DPC markers such as LEF1, Versican, NOG, BMP4, SOX2 and ALP (Figure 24). In Wnt signaling, although it could be observed from the bar graph that the expression of 2D-Exos and 3D-Exos was slightly higher in the LEF1, but according to the statistical results, there was almost no difference compared with the control ($p > 0.05$). The expression of Versican was significantly upregulated when DPCs were stimulated with 3D-Exos and 3D-oxy-Exos. Versican is a member of the aggrecan group involved in matrix assembly and structure as well as cell adhesion [94]. Versican is also a large chondroitin sulfate proteoglycan molecule involved in inducing hair morphology, maintaining hair growth, and initiating hair regeneration. Immunostaining showed that in human skin, Versican was present in the dermis, and was also observed at the dermal–epidermal junction and the connective tissue sheath of the hair follicle [94]. During mouse hair follicle growth cycle, Versican remains negative until the follicle begins to produce fibers. As the follicle

matures, Versican expression in the DP increases, reaches a maximum during the anagen, then gradually declines during the catagen phase and disappears during the telogen phase [94]. K15 is preferentially expressed in the stem cells located in the bulge area and its promoter can target hair follicle bulge cells [95]. Versican protein is almost negatively expressed in the DP in the telogen, whereas Versican protein is deposited outside of K15-positive epithelial cells in the bulge area during the human hair growth cycle. It is reasonable to infer that the Versican protein in the bulge region may decline and enter the DP during the next anagen phase [96]. In patients with AGA, the Versican gene decreases in DP. In a study involving three patients with AGA, more than 15 vellus-like hair follicles in the scalp skin tissue demonstrated that Versican expression was almost lost in the DP in the anagen phase [96].

In BMP signaling, 2D-Exos and 3D-oxy-Exos increased the expression of NOG, with 3D-oxy-Exos increasing more, while 3D-Exos decreased expression of NOG compared with controls. But there was no significant statistical difference between the four ($p > 0.05$). The expression of BPM4 was significantly upregulated when DPCs were stimulated with 3D-Exos and 3D-oxy-Exos. BPM signaling regulates hair follicle proliferation and differentiation. Both BPM2 and BPM4 are activated in hair shaft precursors, and BPM4 is also expressed in the DP [97]. Ectopic expression of BPM4 in outer root sheath (ORS) inhibits proliferation in the hair matrix and activates hair keratin-related genes in ORS [98].

Regarding DP characteristic genes, it was observed that 2D-Exos, 3D-Exos and 3D-oxy-Exos had little change in the expression of SOX2 compared with the control and there was no statistically significant difference. When DPCs were treated with 3D-Exos, the gene expression of ALP was elevated compared with the control. Alkaline phosphatase (ALP) is a zinc metalloenzyme that is primarily anchored to the plasma membrane via a glycosylphosphatidylinositol anchor [99]. The DP exhibits marked ALP activity during and after development, so ALP can be used as a useful marker of DP

location, morphology and size in skin specimens [100]. ALP can be detected in the mesenchymal and epithelial regions of mouse vibrissa follicles. Its positioning and strength can change during the hair cycle. The ALP activity of the DP is moderate in the very early stages of anagen, then gradually rises to a peak in early anagen. After that, ALP activity decreases in the proximal region of the DP after mid-anagen, and remains low during catagen. During catagen phase, the outermost layer of the bulbar epithelium becomes positive for ALP, which may be the precursor of follicular epithelium migrating from the bulge. Before the onset of hair formation, ALP activity in the bulb epithelium rapidly decreases, whereas ALP activity in the DP increases. These changes in ALP activity during the hair growth cycle may be related to the function of DP in hair induction and the remodeling of hair follicle structure [101].

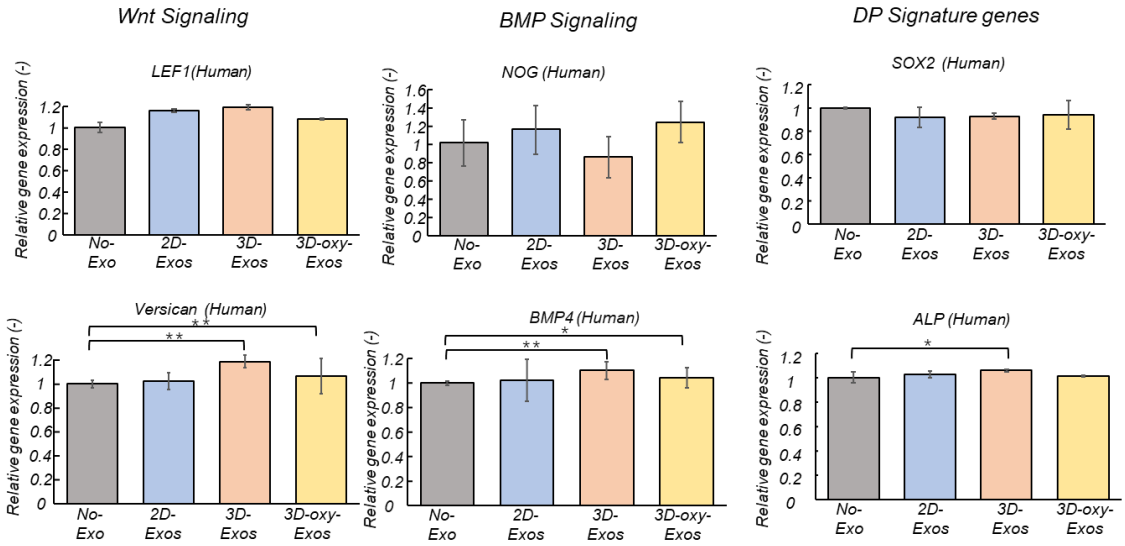

Figure 24. Expression of hair-related genes of DPCs after exosomes or PBS treatment

From the results of these gene expression, the exosomes obtained using the 3D and 3D-oxy cultures have a favorable effect on DPCs *in vitro*. However, in other studies, the differences in gene expression between the exosome-treated group and the control group were more pronounced compared to the present results. For instance, in the study by Kwack et al. [69], the gene expression of SOX2 treated with DP exosomes was about 2.8-fold higher than that of the control, while that of ALP was about 1.8-fold. After analyzing

the reasons, one possible reason is that DPCs of passage 4 was used to prepare exosomes, and the number of passages may affect the activity of cells and thus the activity of exosomes. Another possible reason is that the effect of DPCs-derived exosomes on DPCs mainly focuses on autocrine, which may be difficult to observe directly through DPCs, or the detection of gene expression in this part was not appropriate. Therefore, in future research, methods to solve these two problems need to be explored. One solution is to use more viable and vigorous DPCs to prepare exosomes. Another way to improve is to find a suitable method to evaluate the effect of DPCs after DPC-Exo treatment.

3.3.9. Theoretical basis

Previous studies on the effect of exosomes on hair follicle epithelial cells or their subtypes of HFSCs provide a theoretical basis. DPCs are a unique population of mesenchymal cells that regulate hair follicle development and the hair growth cycle through reciprocal interactions with epithelial cells [67][102]. This implies that exosomes secreted by DPCs are employed to communicate with hair follicle epithelial cells in hair follicles *in vivo*. Previous research has shown that exosomes derived from DPCs promoted hair growth in cultured human hair follicles [69]. In addition, DPC-Exos and their component microRNAs, such as miR-22-5p, mediated HFSCs proliferation and differentiation [103]. Therefore, the exosomes secreted by DPCs may act on epithelial cells.

3.3.10. The effect of exosomes on the proliferation of hair follicle epithelial cells

The effects of exosomes derived from DPCs on the proliferation of human hair follicle epithelial cells was investigated. “No Exo” was used as the label of PBS. “Preincubation”

referred to photos taken under the microscope after the cells were pre-incubated for 24 hours and before the treatment of exosomes or the same volume of PBS. The same number of exosomes was added to the culture media, and hair follicle epithelial cells were cultured in a conventional culture dish for 24, 48 to 72 hours. The cells partially covered the surface of culture dish and showed similar morphology. Cell coverage was higher in exosomes-added wells compared to control wells (Figure 25).

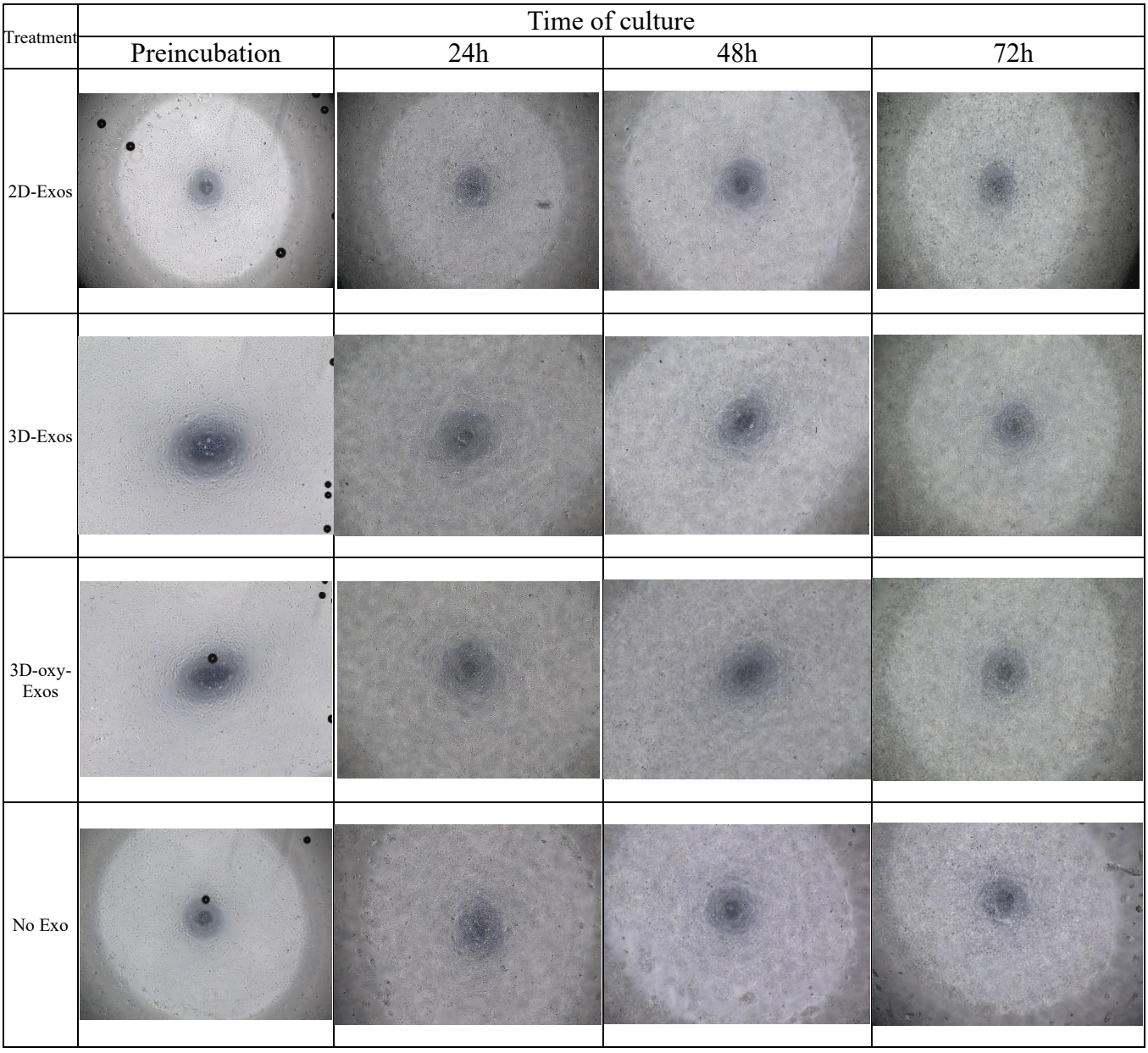

Figure 25. Phase-contrast microscopic images of hair follicle epithelial cells (4×)

Slight increase in cell proliferation was observed in the exosomes prepared from all the culture methods compared with that without exosome supplementation. Namely, irrespective of DPC culture methods, the supplementation of exosomes increased the proliferation of hair follicle epithelial cells (Figure 26), which is consistent with previous reports. (24 h, No Exo vs. 2D-Exos: p=0.428; No Exo vs. 3D-Exos: p=0.501; No Exo vs. 3D-oxy-Exos: p=0.257; 2D-Exos vs. 3D-Exos: p=0.999; 2D-Exos vs. 3D-oxy-Exos: p=0.980; 3D-Exos vs. 3D-oxy-Exos: p=0.953. 48 h, No Exo vs. 2D-Exos: p=0.359; No Exo vs. 3D-Exos: p=0.242; No Exo vs. 3D-oxy-Exos: p=0.167; 2D-Exos vs. 3D-Exos: p=0.992; 2D-Exos vs. 3D-oxy-Exos: p=0.950; 3D-Exos vs. 3D-oxy-Exos: p=0.995. 72 h, No Exo vs. 2D-Exos: p=0.237; No Exo vs. 3D-Exos: p=0.139; No Exo vs. 3D-oxy-Exos: p=0.053; 2D-Exos vs. 3D-Exos: p=0.994; 2D-Exos vs. 3D-oxy-Exos: p=0.922; and 3D-Exos vs. 3D-oxy-Exos: p=0.995.)

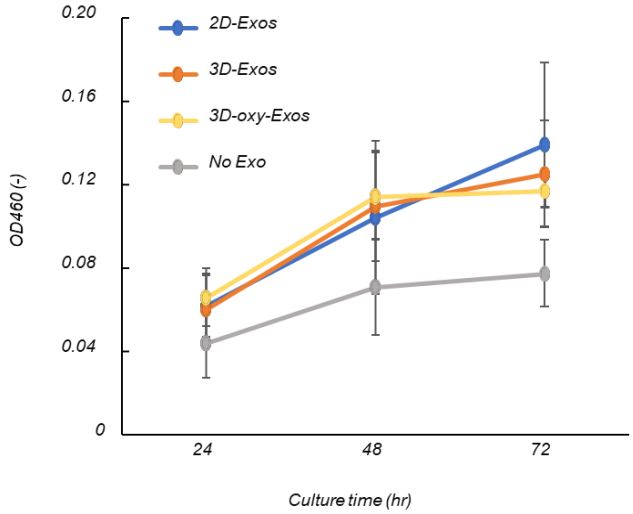

Figure 26. The effects of exosomes on the proliferation of human hair follicle epithelial cells

3.3.11. The genetic impact of exosomes on hair follicle epithelial cells

The effects of exosomes from DPCs on the HFSC marker gene expression in hair

follicle epithelial cells were investigated (Figure 27). Among the stem cell markers, CD200, CD34, and K15 expression was significantly upregulated by supplementation with 3D-oxy-Exos, and there was no difference in gene expression of ITGA6 and K14.

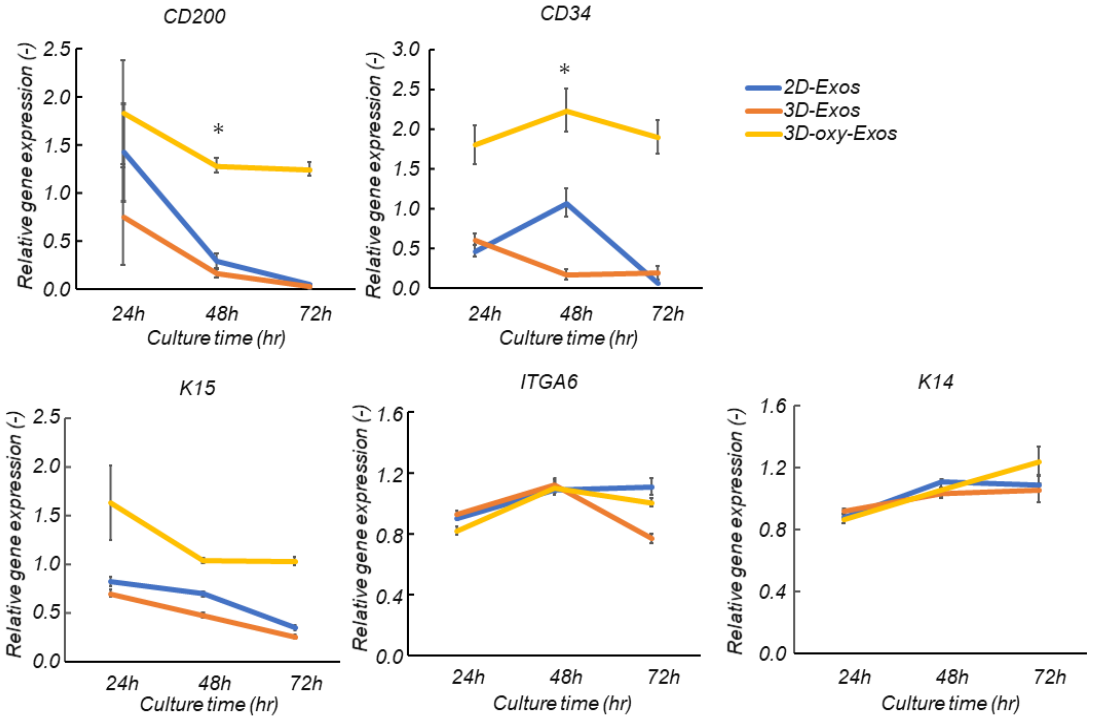

Figure 27. Expression of hair-related genes of hair follicle epithelial cells after exosomes or PBS treatment

Maintenance of stemness and differentiation of hair follicle epithelial cells or their subtypes of HFSCs was investigated using HFSC markers such as CD200, CD34, K15, K14 and ITGA6, and the reasons behind it are as follows. CD200 is a typical HFSC marker found in the bulge [104]. CD200 is involved in the regulation of the immune privileged state of hair follicles [104] and is closely associated with the inhibition of autoimmune inflammation in hair follicles [105]. Because hair loss, such as alopecia areata, lichen planus, and even AGA, is often implicated in the immune response and inflammation, the upregulation of CD200 expression may lead to new therapeutic approaches. Although K15, K14, and α6-Integrin (ITGA6, also known as CD49f) are

expressed in HFSCs [106][107], CD34 is not a direct marker for HFSCs and is identified in cells located in the lower outer root sheathe of the anagen follicle, in the sub-bulge area, and in melanocyte stem cell subpopulations [108][109]. Figure 27 shows that the expression of CD200, CD34 and K15 was significantly upregulated only when hair follicle epithelial cells were stimulated with 3D-oxy-Exos. This is probably because the content and component of exosomes such as mRNA, microRNA, and proteins, are different in 3D-oxy-Exos because of autocrine stimulations. In the microwell array device, oxygen is supplied not only from the top through the culture media but also from the bottom through the PDMS substrate. Therefore, DPC spheroids were cultured in a high-density arrangement. Based on the exosome secretion rate in Figure 20C and the ratios of the cell number to medium volume (2.0×10^5 cells, 10 mL in 2D culture, 2.0×10^5 cells, 6.5 mL in 3D culture, 2.0×10^5 cells, 0.4 mL in 3D-oxy culture), the concentration of exosomes was 2.45 times higher in 3D-oxy culture than in 2D and 3D cultures. Autocrine stimulation wherein exosomes derived from human DPCs augmented the hair-inductive capacity of DPC spheroids has been shown [69].

3.3.12. Previous studies on whether human cell-derived exosomes are effective for hair growth in mice

In preliminary observational experiments of efficacy, mouse cells or tissues are easier to obtain or manipulate than human ones. In the present study, human DPCs were used to prepare exosomes. As for whether human cell-derived exosomes are effective in mice, there are relevant articles to support. Some studies have been involved in the effect of exosomes derived from human DPCs on the hair growth effect of mice. The study by Kwack et al.[69] evaluated the effect of human 3D DP-Exo on hair growth after subcutaneous injection into mice. The dorsal skins of 35-day-old female C57BL/6 mice in anagen were shaved and injected once daily for 4 days with PBS or 50 μg of 3D DP-

Exo. On day 40, according to HE staining, the hair follicles of the skin injected with 3D DP-Exo were in the anagen, whereas those injected with PBS were in the catagen. Besides, on day 42, the hair follicles treated with exosomes were still in the anagen, but those injected with PBS were in the telogen. Next, the dorsal skins of 49-day-old female C57BL/6 mice in telogen were shaved and injected 3 times a week with PBS or 50 μg of 3D DP-Exo for 26 days. According to HE staining results on days 77 and 79, injection of 3D DP-Exo accelerated the anagen, while mice injected with PBS were still in telogen. After that, 3D DP-Exos or PBS were used to treat human DP spheres, which were then co-transplanted with epidermal cells from fetal mice. More Hair follicles were formed in exosome-treated groups than in PBS groups. 3D DP-Exos increased gene expression in the Wnt and BMP signaling pathways, and significantly increased the expression of DP signature genes such as SOX2, Hey1, and ALP. To sum up, it was found that human 3D DP-Exo can prolong the anagen phase of mouse hair follicles and promote the telogen phase to enter the anagen phase more quickly. In addition, when human DP spheres were treated with human 3D DP-Exos and then co-implanted with mouse epidermal cells, hair follicle induction was significantly greater than those treated with PBS. Another study by Zhou et al. [67] injected human 2D cultured DPC-Exos into dorsal skins of C57BL/6 mice in telogen and anagen phase, while control mice were injected with PBS. HE staining analysis showed that the hair follicles treated with DPC-Exos had healthier morphology, larger size and more numbers than the control groups. The results, after evaluation, showed that human DPC-Exo injection in the skin of mice in anagen delayed the onset of catagen. Higher levels of immunostaining positive for β-catenin in the ORS and DP were observed in DPC-Exo-treated mice groups. Shh-positive cells were mainly detected in the bulge area, and the DPC-Exo-treated groups also showed higher expression of Shh than the control groups. In conclusion, these results suggested that human DPC-Exos have anagen-promoting activity in the mouse hair follicle growth cycle.

3.3.13. Effects of exosomes on hair follicle sprouting

Mouse embryonic epithelial and mesenchymal cells were isolated from mouse fetuses, from which a mixed cell suspension solution was seeded in a 96-round-bottom-well plate. The cells formed cell aggregates of 500 μm in diameter in three days of culture regardless of adding exosomes or PBS (Figure 28A). Hair follicle sprouting was observed *in vitro* with or without exosome supplementation. Note that the DP was externally oriented, and the hair shaft was inwardly oriented in the aggregates (Figure 28A). Significantly more hair follicle sprouting was observed when 2D-Exos and 3D-oxy-Exos were used as supplements. In contrast, no statistically significant increase in the sprouting was observed with 3D-Exos compared with that in the PBS control (Figure 28B). The analysis of trichogenic gene expression in hair follicle organoids revealed that Versican and TGFβ2 expression was slightly upregulated and significantly downregulated, respectively, by supplementation with 3D-oxy-Exos (Figure 29). In addition, IGFbp5 expression was significantly upregulated by 3D-oxy-Exos supplementation. No obvious changes were observed in the Wnt10b expression.

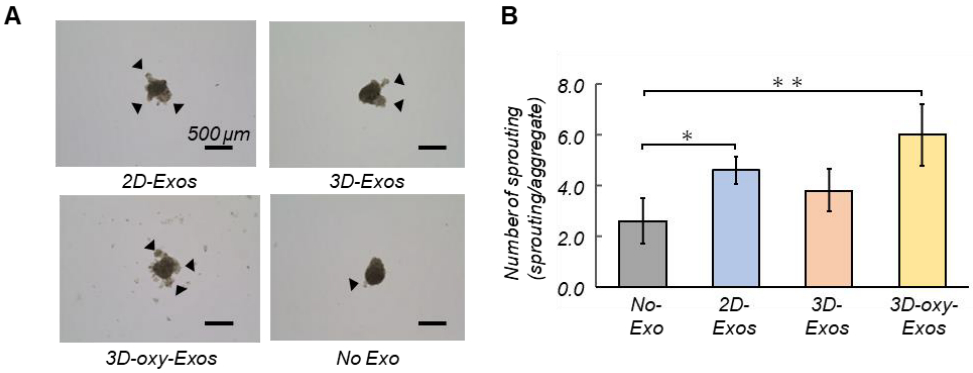

Figure 28. Effects of exosomes on hair follicle organoids formation

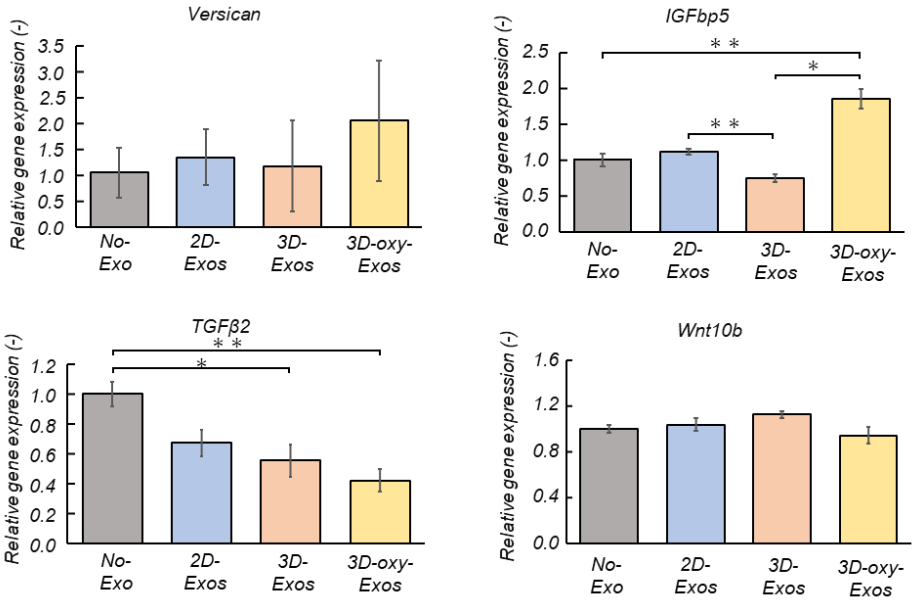

Figure 29. Trichogenic gene expression of hair follicle organoids after exosome treatment or PBS treatment

This study also showed that exosomes derived from human DPCs stimulated mouse hair follicles to prolong the anagen phase and promote phase changes from the telogen to anagen. In the present study, to further investigate the effects of exosomes from human DPCs, hair follicle organoids were prepared with mouse embryonic epithelial and mesenchymal cells and exposed to exosomes from three culture methods. 3D-oxy-Exos significantly increased hair follicle sprouting (Figure 28B), upregulated IGFbp5 expression, and downregulated TGFβ2 expression in the hair follicle organoids (Figure 29). Among the six high-affinity insulin-like growth factor-binding proteins (IGFbps), IGFbp3 and IGFbp5 are mainly expressed in DPCs [110]. IGFbp5 expression changes throughout the hair growth cycle. IGFbp5 is expressed in cells of the entire dermis, subcutaneous tissue, and in the DP of anagen hair follicles. IGFbp5 has been reported to enhance the effect of insulin-like growth factor I (IGF-I), which plays a key role in hair growth and development [111]. Co-expression of IGFbp5 and IGF-I in DPCs has been revealed by *in situ* hybridization of anagen hair follicles [110]. Furthermore, IGFbp5 has been reported to affect in the interaction of extracellular matrix proteins to regulate the remodeling of extracellular matrix tissue [112]. These findings indicate that IGFbps play

an important role in epidermal–mesenchymal interactions, and participate in the regulation of hair growth and cell differentiation. Moreover, in the physiology of hair follicles, the TGFβ family have both promoting and inhibiting effects on hair growth. TGFβ1 and TGFβ2 stimulate the proliferation of outer root sheath keratinocytes [113]. The upregulation of TGFβ2 expression in cultured DPCs enhanced their hair-inducing ability and inhibition of TGFβ2 signaling at the ligand and receptor levels largely inhibited hair follicle generation and maturation in an animal model [114]. However, a negative effect manifests in the hair growth cycle. At the onset of catagen, DHT stimulates the synthesis of TGFβ2 in the DP, and TGFβ2 then stimulates the synthesis of caspases. TGFβ2 triggers the intrinsic caspase network, resulting in the elimination of epithelial cells via apoptotic cell death. The use of TGFβ antagonists has been shown to promote hair follicle elongation and prevent changes in hair follicles, such as in the catagen phase, *in vivo* and *in vitro* [115]. It is obvious that further investigations are necessary to elucidate the mechanisms responsible for the effects of exosomes on the maintenance of hair follicle epithelial cells and the promotion of hair follicle sprouting. This includes a detailed analysis of the components of exosomes and their effects on trichogenic gene expression and signaling pathways.

3.4. Conclusion of this chapter

In this chapter, DPC-secreted exosomes were selected for the study of extracellular signaling of DPCs responsible for hair induction. Exosomes were collected from human DPCs in three different culture methods: 2D culture, 3D culture and 3D-oxy culture in the oxygen-permeable PDMS microwell array plate. Initially, ultracentrifugation and polymer precipitation methods were used for the preparation of exosomes. However, after the protein concentration experiment of exosomes was carried out, the concentration of exosomal protein obtained by ultracentrifugation method was lower than that of polymer precipitation method. Besides, the operation steps of ultracentrifugation method were relatively complicated and special instrument support was required. Therefore, polymer

precipitation method was chosen to be used to prepare exosomes. Since exosomes are barely visible, it is necessary to demonstrate that the prepared particles are exosomes and do not contain cellular contamination. Due to this reason, the size and shape of three kinds of exosomes were examined, as were their amount and surface antibodies. According to the results in this chapter, the prepared particles conformed to the characteristics of exosomes and contained little cellular contamination.

Purified exosomes were exposed to human DPCs and hair follicle epithelial cells as well as hair follicle organoids composed of mouse embryonic epithelial and mesenchymal cells. This study found that irrespective of DPC culture methods, the supplementation of exosomes increased the proliferation of DPCs. Compared with the control (same volume of PBS), 2D-Exos can promote the proliferation of DPCs at 48 hours and 72 hours; 3D-Exos and 3D-oxy-Exos can also promote the proliferation of DPCs at 72 hours, but not as obvious as 2D-Exos. However, after statistical calculation, the difference was not significant. Maintenance of key roles of DPCs was investigated with DPC markers such as LEF1, Versican, NOG, BMP4, SOX2 and ALP. The expression of Versican and BPM4 was significantly upregulated when DPCs were stimulated with 3D-Exos and 3D-oxy-Exos. When DPCs were treated with 3D-Exos, the gene expression of ALP was slightly elevated compared with the control. From the results, the exosomes obtained using the 3D and 3D-oxy cultures have a favorable effect on DPCs *in vitro*.

Effects of DPC-Exos on hair follicle epithelial cells proliferation were examined. Irrespective of DPC culture methods, the supplementation of exosomes increased the proliferation of hair follicle epithelial cells, which is consistent with previous reports. Maintenance of stemness and differentiation of hair follicle epithelial cells represented by HFSCs was investigated using HFSC markers such as CD200, CD34, K15, K14 and ITGA6. The results showed that the expression of CD200, CD34 and K15 was significantly upregulated only when hair follicle epithelial cells were stimulated with 3D-oxy-Exos.

To further investigate the effects of exosomes from human DPCs, hair follicle

organoids were prepared with mouse embryonic epithelial and mesenchymal cells and exposed to exosomes from three culture methods. 3D-oxy-Exos significantly increased hair follicle sprouting, upregulated IGFbp5 expression, and downregulated TGFβ2 expression in the hair follicle organoids. It showed that exosomes derived from human DPCs stimulated mouse hair follicles to prolong the anagen phase and promote phase changes from the telogen to anagen.

According to these results, 3D-oxy-Exos had the best hair growth promotion effect. This is probably because the mRNA, microRNA, and proteins are different in 3D-oxy-Exos because of autocrine stimulations of DPCs. In the microwell array device, oxygen is supplied not only from the top through the culture media but also from the bottom through the PDMS substrate. Therefore, DPC spheroids were cultured in a high-density arrangement. Based on the exosome secretion rate, the concentration of exosomes was 2.45 times higher in 3D-oxy culture than those in 2D and 3D culture. Autocrine stimulation wherein exosomes from human DPCs promoted the hair-inducing ability of DPC spheroids, thereby enhancing extracellular signaling and paracrine effects on surrounding cells or tissues.

CHAPTER 4

4. Thesis summary and conclusion

Chapter 1 began with a brief introduction to hair and its importance to humans. The hair growth cycle and the major cells involved in it were then elucidated. The disease of hair loss was introduced. Among the hair loss diseases, AGA is the most common and has seriously troubled modern people. The basic disease characteristics, age of onset and sex predisposition, as well as etiology of AGA were described. Hair loss treatments and their advantages and disadvantages were then introduced. In cell therapy, *in vitro* culture of DPCs is a hot topic. As another research hotspot in recent decades, exosomes have good development prospects, whether in the field of dermatology or other fields. Exosomes have also been studied in the field of hair. Finally, Chapter 1 concluded with the purpose of this study.

In Chapter 2, the trichogenic gene expression was significantly upregulated in HBs, but downregulated in 2D culture. Transcriptome comparison showed that PI3K/Akt signaling pathway plays an important role in promoting hair growth. Inhibition of the PI3K/Akt signaling pathway decreased the hair-inducing capability of DPCs, while the activation of the PI3K/Akt signaling pathway improved gene expression of DPCs. It suggests that the PI3K/Akt is the underlying intracellular signaling pathways of DPCs responsible for hair induction, which is instructive for remodeling and maintaining DPC function *in vitro*.

In Chapter 3, DPC-secreted exosomes were selected for the study of extracellular signaling of DPCs responsible for hair induction. 2D, 3D and 3D-oxy cultures were used to prepare exosomes from human DPCs. After ensuring successful harvest and purification of exosomes, the effect of exosomes on hair growth was investigated. From the results as mentioned above, the exosomes obtained using the 3D and 3D-oxy cultures

had a favorable effect on DPCs *in vitro*. 3D-oxy-Exos enhanced maintenance of stemness and differentiation of hair follicle epithelial cells or HFSCs *in vitro*. 3D-oxy-Exos stimulated mouse hair follicles to prolong the anagen phase and promote phase changes from the telogen to anagen. The reason behind the performance of 3D-oxy-Exos over the other two may be due to autocrine stimulations, the content and component of exosomes in 3D-oxy-Exos (including mRNA, microRNA and proteins) are different. Moreover, in the microwell array device, oxygen is supplied from the top through the culture media and from the bottom through the PDMS substrate. Therefore, in 3D-oxy culture, DPC spheroids show a high-density arrangement state, resulting in better function of 3D-oxy-Exos. Autocrine stimulation of exosomes from human DPCs has promoted the hair-inducing ability of DPC spheroids, thereby enhancing extracellular signaling and paracrine effects on surrounding cells or tissues.

Chapter 4 is the summarization of the thesis.

Acknowledgements

More than two and a half years have passed since I entered Japan on September 25, 2019. After entering Japan, I started my doctoral career at Fukuda Lab of Yokohama National University in October of that year. For me, who had only traveled abroad for a few days, this study abroad career was a brand-new experience. In China, I completed my undergraduate and master's courses in clinical medicine. During my master's degree, I worked as a doctor in a hospital for 3 years. After coming to Japan, I faced a new country, a new language, new people and a new field of scientific research that was different from clinical practice. Therefore, I have been able to get to where I am today, sitting in front of the computer and writing my doctoral thesis, based on the help of too many people.

First of all, I am greatly indebted to my supervisor, Prof. Junji Fukuda. When I first came to the laboratory, I was very apprehensive when faced with a new environment. Although Prof. Fukuda is very busy, he is very kind and helpful. Whether it is the admission procedures, the dormitory arrangement, the scholarship application, the establishment of the research direction, and the discussion of the research content, Prof. Fukuda has given me selfless help and support in all aspects. After I have carried out the experiment, Prof. Fukuda often gives me suggestions and opinions and helps me establish the experimental direction of each step. I am also very grateful to Prof. Fukuda for his careful reading of the manuscript and my thesis as well as valuable instructions and suggestions on them.

Besides, I would also like to thank Prof. Kazuyuki Hiratsuka, Prof. Atsushi Suzuki, Prof. Kazutoshi Iijima, and Prof. Tadashi Nittami of Yokohama National University for their kind guidance and advice during thesis defense.

My sincere thanks are also given to Prof. Kanda and Mr. Hirata from the National Institute of Health Sciences for helping me in the early stages of my experiment. When I was initially faced exosomes, they provided an ultracentrifuge to help me prepare

exosomes and taught me many experimental methods and procedures.

Around April 2020, because my research direction was hair, Prof. Fukuda arranged for me to study and do research in the laboratory of the Life Innovation Center (LIC) in Kawasaki. So I would also like to thank everyone at LIC. First of all, I would like to thank the assistant professor Dr. Tatsuto Kageyama, who has given me a lot of help and support in experiments. Because before coming to Japan, I mainly studied clinical medicine, and my foundation for experimental research was very weak. Dr. Kageyama taught me a lot of experimental methods, and showed me the way when I had no clue about the next experiment. When my phased experiment failed, he also actively helped me explore the reasons and find solutions. Because of his continued help, my experiments have been gradually on the right track. At the same time, I would also like to thank my labmates, Miss Monami Yamane, Miss Ayaka Nanmo, Mr. Kohei Suzuki, Miss Yumeng Wu, and Miss Shan Tu. Many specific experimental operations are inseparable from their help and guidance. Especially Miss Yamane, she was also one of my deskmates, and I have consulted her on many details in my experiments. Also, I would like to thank my other deskmate, Mr. Ryuji Tachibana. He is enthusiastic and helpful. He and I exchanged a lot of Japanese culture and improved my Japanese expression skills.

Moreover, I feel grateful to all the professors, teachers, seniors, secretary and labmates in Fukuda Lab for their care and help in my doctoral career. I also thank them for their valuable suggestions that have made this thesis a reality. Fukuda Lab is like a big warm family, facing difficulties together, solving problems together, and sharing happiness together.

Last but not least I owe much to my parents who are far away in China but have always given me spiritual care and support, as well as financial help. Their care and support has allowed me to concentrate on my studies and research in Japan.

Because of the coronavirus epidemic, many things have been affected, whether in our daily life, or our study and work. Presumably my study abroad experience is different from other international students before the outbreak of coronavirus. But I think life is

about adjusting yourself as soon as possible and moving forward even if unexpected things happen. After coming to Japan, thanks to everyone's help, I am able to get here along the way. This study abroad experience will definitely be a good memory in my life.

In the end, there is an ancient Chinese poem that is my motto, so please allow me to share it with you. David Hawkes translated it as " Long, long had been my road and far, far was the journey; I would go up and down to seek my heart's desire."

The original text is "路漫漫其修遠兮，吾將上下而求索."

2022 年 9 月 Zhou yinghui 周 迎慧

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