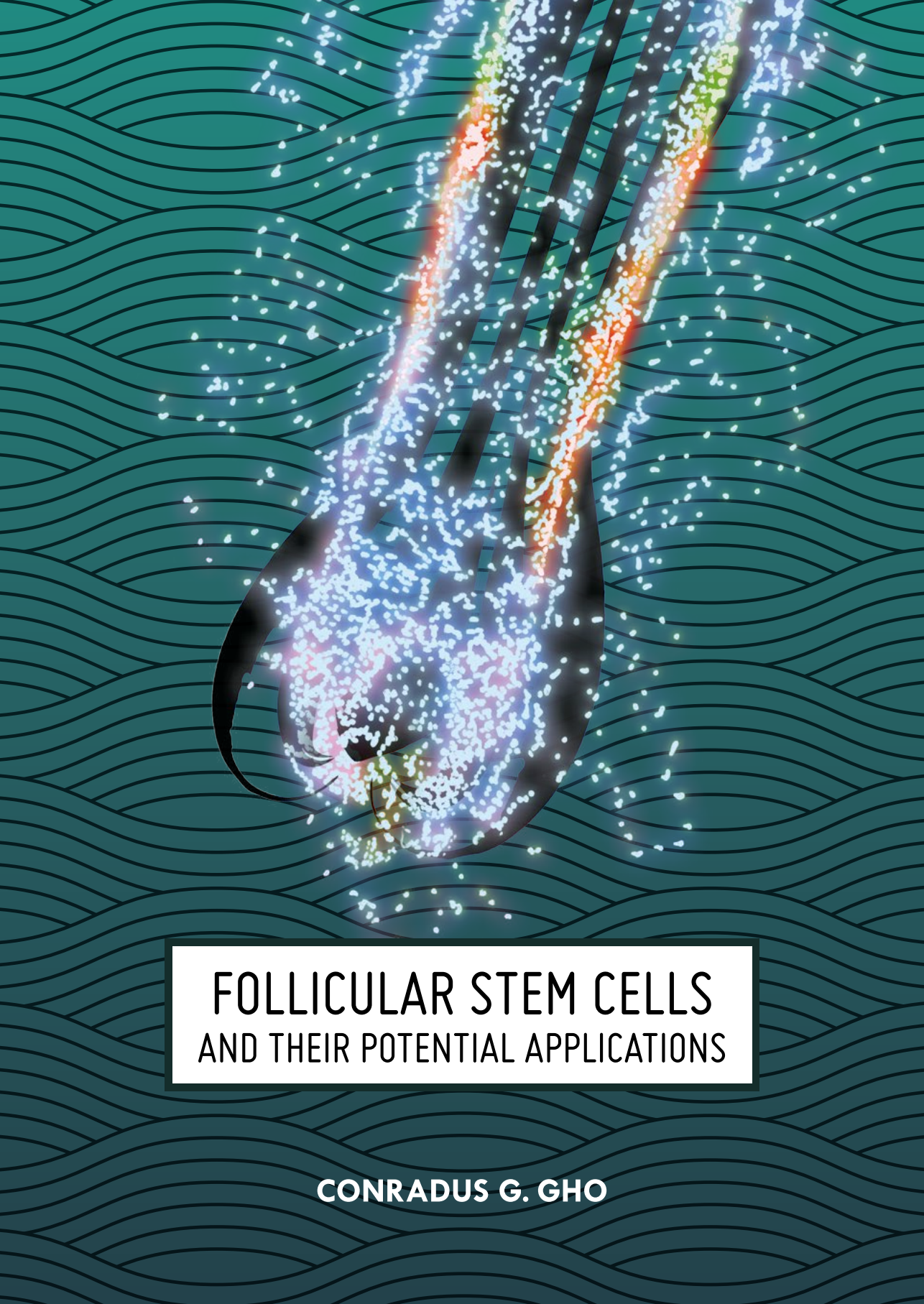

FOLLICULAR STEM CELLS
AND THEIR POTENTIAL APPLICATIONS

CONRADUS G. GHO

“Haarstamcellen en hun mogelijke toepassingen”

“Follicular stem cells and their potential applications”

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“Follicular stem cells and their potential applications”

Proefschrift
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“Sometimes, the smallest things take up the most room in your heart”

– Winnie-the-Pooh

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GENERAL INTRODUCTION
AND AIMS OF THE THESIS

1. GENERAL INTRODUCTION

Adapted from Gho, C. G. and H.A.M. Neumann (2011). "[Therapeutic options for androgenetic alopecia]." Ned Tijdschr Geneeskd 155: A2535.

Hair diseases are more common than many physicians are aware of. These diseases are caused by numerous factors such as genetic factors, hormonal abnormalities, inflammatory diseases and injuries. The physiology of the hair follicle and the growth characteristics are imperative in understanding the problems encountered in the diagnosis and the treatment of hair diseases.

Anatomy of the hair

The microscopic structure of the hair can be subdivided into several structures (from the inside out) as shown in Figure 1:

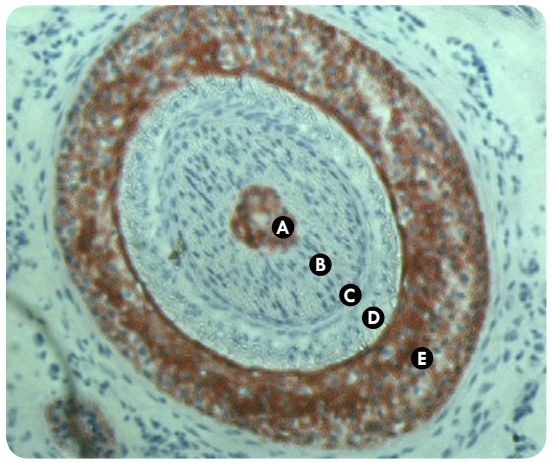


Figure 1: Transverse cross-section of a hair follicle.

(A) Medulla: Marrow
present in both internal and external hair

(B) Cortex: Fiber layer
present in both internal and external hair

(C) Cuticula: Scale layer
present in internal and external hair

(D) Inner root sheath: Innermost layer of the hair sheath
present only in the internal hair part of a hair follicle

(E) Outer root sheath: Outermost layer of the hair sheath
present only in the internal hair part of a hair follicle

There are two important areas in the hair follicle which are shown in Figure 2:

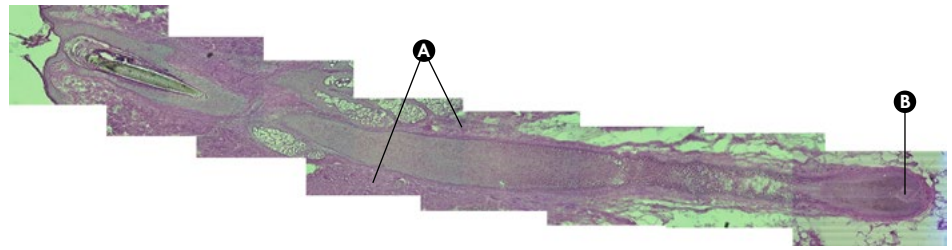


Figure 2: A longitudinal cross-section of a hair follicle.

(A) Bulge-area: Part of the outer root sheath under the sebaceous gland.
Present only in the internal hair part of the hair follicle.

(B) Dermal papilla: This part is also called the hair bulb or the hair papilla. The dermal papilla contains two capillary vessels.
Present only at the base of the internal hair part of the hair follicle.

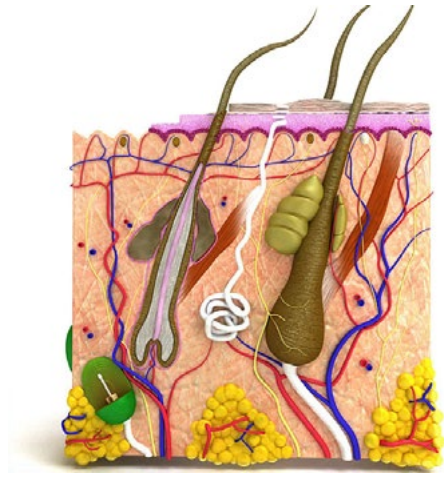


Figure 3: A longitudinal cross-section of a hair follicle.

Furthermore, there are the so-called hair-annexes (= appendices) such as the hair muscle and the sebaceous gland (Fig. 3). The hair muscle ensures that the hair remains intact under certain conditions such as cold temperatures. The result is goose bumps. The sebaceous glands are responsible for the greasiness of the hair and the skin.

Composition of the hair
The composition of the hair denotes the different substances that are present in the hair. The hair consists of endosperms (±85%), water (±12%), greases (±3%) and trace elements (±1%) such as zinc, lead, copper and selenium. The water percentage is doubled when the hair is dropped into water. Furthermore, the percentage of water depends on the air humidity, the greasiness and the hair structure.

Hair color
The color of the hair depends on the cells producing the pigment. There are three types of pigments:

- Eumelanin = blond/light brown/dark brown
- Pheomelanin = red/purchaser gold/ auburn
- Trichosiderin = containing iron pigment substance and is red

The composition and the production of the different types of the pigments depend on the genetic constitution resulting in different hair colors, which can vary from white/high blond to dark black. It is possible that the production and the composition of the pigments vary during life resulting in a chance that one has high blond hair during childhood, dark brown hair at a higher age and gray to white hair late in life.

Hair texture
The skin between the hair follicle and the surface of the skin is stipulating the form of the hair (straight, wavy or curly). When the hair root canals are bent in this part, it may result in wavy or curly hair. In certain periods such as childhood and puberty, it is possible that the flexibility and the stiffness of the scalp skin varies during other life periods. Thus, it is possible that during childhood one has wavy hair, during puberty straight hair and during adulthood straight hair once again.

Classification of the hair (Fig. 4)
The usual classification of hair types is:

- Lanugohair: Silky hair without pigment that is usually ricocheted in the uterus, but sometimes is still present on the skin of a new-born.
- Vellus hair: Thin short hair without pigment
- Terminal hair: Thick coarse hair of different lengths that is generally pigmented

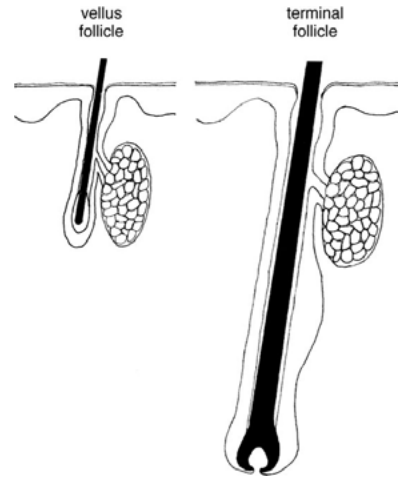


Figure 4: A comparison of vellus and terminal hair

Beside this classification, the existence of terminal hair on the body is also directed by hormones:

1. Growth is independent on the sensitivity of the male hormones. Examples:

- Back side of the scalp
- Eyebrows
- Lashes
- Nose hair
- Ear hair

2. Growth depends on the sensitivity of the male hormone testosterone and to a lesser degree on the male hormone dihydrotestosterone. Examples:

- Armpit hair
- Pubic hair

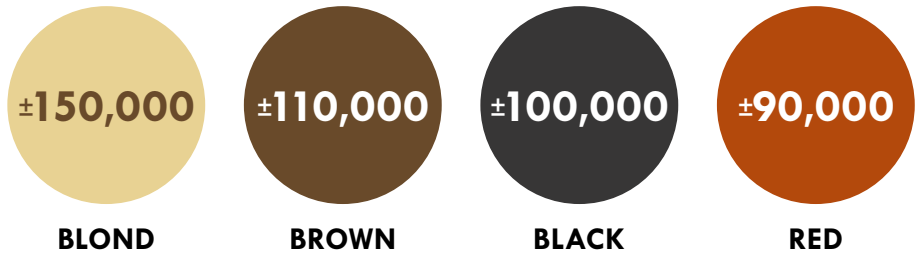
3. Growth depends on the sensitivity of the male hormone dihydrotestosterone, but to a lesser degree on the male hormone testosterone. Examples:

- Beard region
- Hair on the limbs
- Chest hair and hair on the back

1

Density of the hair

The normal density of hair follicles varies between ± 1000 hair per square centimeter in babies to 250 hairs in individuals aged around 50 years. The total number hair on the scalp varies between 90,000 and 150,000 hairs. This varies by hair color:



Density of the hair

The hair cycle consists of three phases (Fig. 5). The first phase is the anagen or growing phase. This phase can vary from a month (eyebrows) up to 12 years (scalp) depending on the location. The differences in the life span explains directly the differences in the length in the different areas of the body (Table 1). The catagen or the passage phase follows. The hair grows no more at this stage and it starts to die. Finally it ends up in the telogen or the rest phase. This phase lasts approximately 3 to 6 months. Since this phase lasts so long, it explains why the hair loss takes place just 3 to 6 months after an (adverse) event.

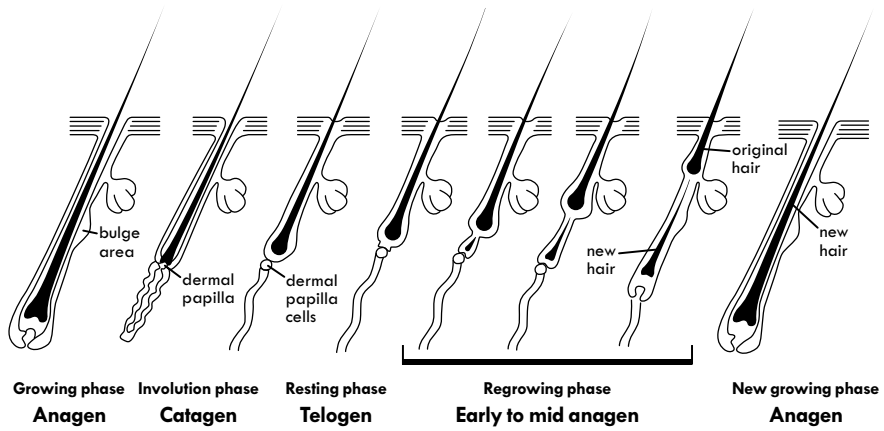


Figure 5: Hair cycle.

1

The rate of hair growth (Table 1)

Depending on the location, hair grows an average of 0.1 mm/day (body hair) up to 1mm/day (beard region). Between the different individuals however large differences can exist. This is caused by the genetic properties of the individual.

Types of hair		Anagen	Catagen	Telogen	Growth rate	Maximum length
Type I	Scalp	10 – 12 years		3 – 6 months	0.44 mm/day	100 – 120 cm
	Eyebrows	3 – 6 months		3 – 6 months	0.1 mm/day	1 – 2 cm
	Lashes	1 – 2 months		3 – 6 months	0.1 mm/day	0.5 – 1.0 cm
	Nose hair	10 – 12 yrs		3 – 6 months	0.1 mm/day	0.5 – 1.0 cm
	Ear hair	10 – 12 yrs		3 – 6 months	0.1 mm/day	0.5 – 1.0 cm
Type II	Armpit hair	10 – 12 yrs		3 – 6 months	0.3 mm/day	4 – 6 cm
	Pubic hair	10 – 12 yrs		3 – 6 months	0.3 mm/day	6 – 8 cm
Type III	Barbe region	10 – 12 yrs		3 – 6 months	1 mm/day	75 – 100 cm
	Hair on the limbs	10 – 12 yrs		3 – 6 months	0.44 mm/day	2 – 3 cm

Table 1: Characteristics of different types of hair.

The differences between plucked hair and a whole hair follicle

Both the plucked hairs as well as the whole hair follicles were used in the reported studies. The differences between a plucked hair (Fig. 6) and a whole hair follicle (Fig. 7) is that plucked hairs only contain epithelial structures such as the inner- and outer-root sheaths as well as a part of the dermal papilla, but do not contain (a part) of the annexes and most of the dermal tissue. The whole hair follicle contains, besides an inner- and outer root sheath, the whole dermal papilla and (a part) of the annexes and most of the dermal tissue.

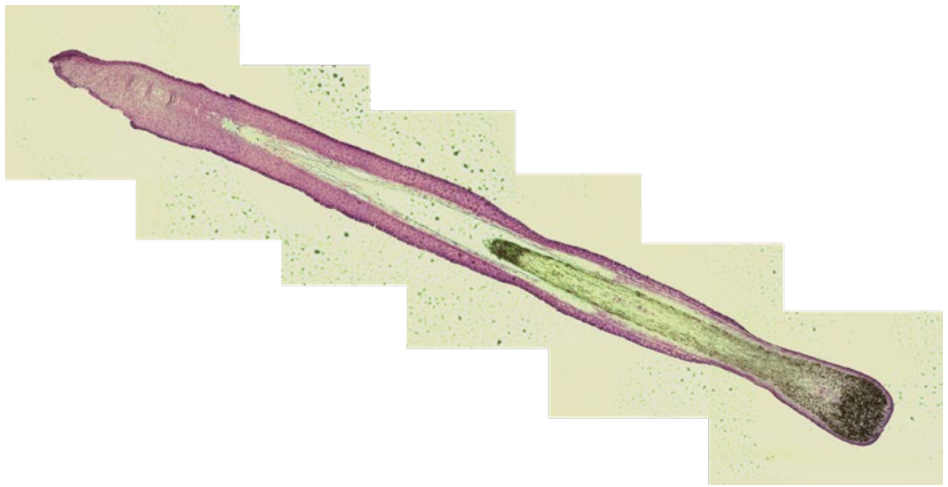


Figure 6: A longitudinal cross section of a plucked hair.

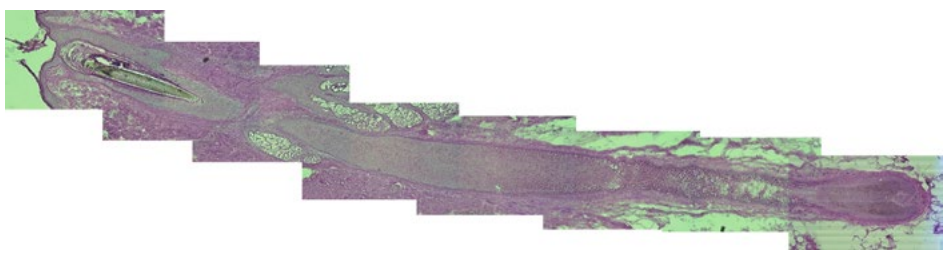


Figure 7: A longitudinal cross section of a whole hair follicle.

Androgenetic Alopecia

Hair loss is a common problem with different underlying causes (Table 2). Androgenetic alopecia (AGA) is the most common form. It involves the familiar male pattern of hair loss (Fig. 8). The prevalence of AGA at the age of 50 years is about 50% for Caucasian men and 30% for Caucasian women.[1,2] For Asians, American Indians and African-American men the prevalence of AGA is lower.[3] Androgenetic alopecia has a genetic predisposition and polymorphism has also been discovered in the gene of the androgen receptor, but not all genes responsible have yet been identified.[4,5]

Figure 8: Androgenetic Alopecia in the author.

Hair loss characteristics			Examples	Duration of symptoms
Objective	Scarred	Inflammatory	Lichen planus, lupus erythematosus, folliculitis decalvans, classic Brocq's pseudopelade	Years – lifetime, progressive
		Physical	Burn wounds, irradiation, accident	Lifetime, not progressive
	Non-scarred	Localized	Alopecia Areata	Weeks/months, sometimes years and progressive
		Frontal/Crown	Androgenetic alopecia (AGA)	Years – lifetime, progressive
		Diffuse	Telogen/anagen effluvium, for example as a result of medication, thyroid dysfunction, fever, anesthetic	Weeks, Months, Years, sometimes progressive
Subjective			Psychological most of the times	Weeks – years, not progressive

Table 2: Various forms of hair loss.

In addition to genetic predisposition and hormonal status, other psychological factors such as stress also affect the course of hair loss.[6]

The number of resources and options to prevent and cure AGA are also increasing. The treatment spectrum ranges from doing nothing to surgical intervention. However, the treatment of AGA is by no means simple.

Causes of AGA

Androgenetic alopecia (AGA) is caused by the binding of dihydrotestosterone (DHT) to the androgen receptor in the hair follicle. The enzyme 5-α-reductase, which is located in the hair follicle, converts testosterone into DHT, which has an approximately 10-50 times stronger androgenic effect than testosterone. There are two types of 5-α-reductase, type I and II. In the cranial area of the scalp, type I is mainly present, whereas type II is mainly present in the area of the beard and in the prostate. The relationship between the different types of 5-α-reductase determines whether the hair growth is decreased (cranial area of the scalp) or is in fact stimulated (beard area).[7]

In turn, DHT is dissolved by the enzyme aromatase. The individual differences in the severity and the progression of AGA are, among others, explained by the individual genetic differences in the levels of 5-α-reductase, aromatase and androgen receptor.[7]

Under the influence of androgens the following changes occur:

- The follicle unit produces only 1-2 hairs instead of 3-4 hairs.
- The hair follicles become smaller.
- The anagen phase is shortened.
- The number of hairs in the telogen phase increases.
- The hair diameter decreases.
- The terminal hairs are increasingly replaced by vellus hair, which are soft, marrow-free, non-pigmented, short and barely visible.

The quality of life

Although AGA is a natural process, it is not accepted by everyone, especially when it occurs early in life. The hair problem is even greater in women, because society considers women with alopecia as abnormal. After all, hair plays a major social role. Androgenetic alopecia may be a major psychological problem for both men and women. The quality of life sharply declines with the progression of baldness. Mental depression is more frequent as compared with that in the normal population.[8] Therefore, the complaints of hair loss should be taken seriously both in terms of the emphasis and the objective information.[9,10]

The clinic

The hair on the scalp grows over a period of 6 to 12 years. The hairs are then in the so-called anagen or growing phase. Then, the hair go into the catagenic or intermediate phase for 1-2 months and subsequently go into the telogen or resting phase for another 2 to 4 months after which it falls out. A new (anagen) hair then begins to grow again on the same site.

Androgenetic alopecia manifests itself through an increased loss of scalp hair. The skin of the scalp does not appear to be different. The only observation is a decrease in the follicular size and a total disappearance of the hair follicle in a late stage. Histologically, miniaturization with a variation in the size of the hair follicles and an increased number of vellus hair are diagnostic for AGA.[11] A hair root examination and a pluck test (see explanation box 1. "Criteria for the diagnosis of Androgenetic Alopecia"), which should be performed in a standardized manner (four days after washing), one is able to determine whether the ratio between telogen (falling out) and anagen (growing) hairs, also known as the hair-root status investigation, is disturbed.

The differential diagnosis of AGA in men is very limited due to the pattern of hair loss. However, in women, diffuse effluvium due to hormonal disturbances may be difficult to distinguish from AGA.[12] The diagnosis of 'AGA' is made on the basis of a set of criteria shown in Box 1.

BOX 1: Criteria for the diagnosis of Androgenetic Alopecia

1. A specific pattern of hair loss (Figures 9 and 10).
2. No scarring.
3. Positive hair pluck test: If during the 'plucking' of about 100 hairs at least 10 hairs come out on their own, then it would mean that more hairs are in the telogen (falling out) phase than normal.
4. Positive hair root status investigation: cranially, the number of telogen hair roots is increased in relation to the number of anagen hair roots.

Criteria 1 and 2 are pathognomic for androgenetic alopecia.
Criteria 3 and 4 are additive.

Additional investigations including a biopsy should be carried out in order to exclude other causes of hair loss when in doubt. An underlying hormonal disorder such as polycystic ovary syndrome or hyperthyroidism must be excluded especially in rapidly progressing AGA in women.[13] There is a large biological diversification both in the moment of the first clearly visible signs of alopecia and in its severity. There is a difference between the early AGA before the age of 30 years (AGA Praecox) and the late AGA (from about the age of 50 years) and in women both the pre- and post-menopausal hair loss. The AGA Praecox should be regarded as a disease and no longer as a natural process of the aging body.

Men

A reduction in the hair density on the crown or a receding hairline (on the sides) is the clearest indication of AGA in men. There are several patterns of AGA in men, which are classified according to Norwood-Hamilton as shown in Figure 9.[14]

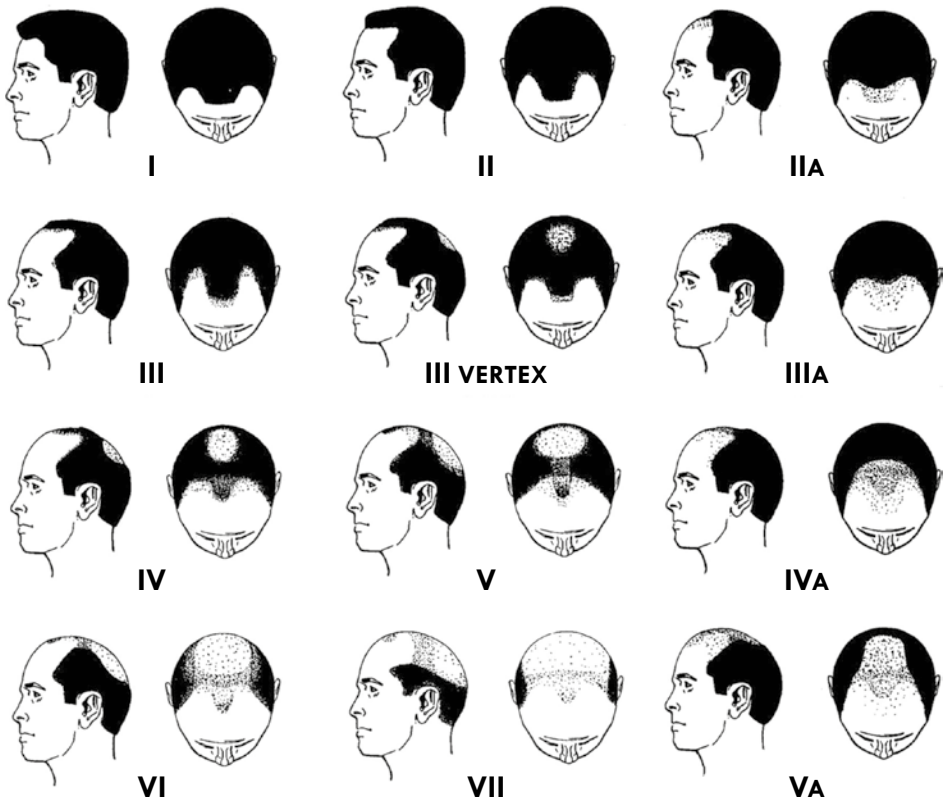


Figure 9: Male AGA classification according to Hamilton/Norwood.[14] Figure from a previous publication.[15]

Women

In women, AGA is primarily the hair density that has decreased cranially, whereas the front hairline has typically remained intact. Unlike men, women usually do not become completely bald locally, but the density of hair is reduced and the remaining hairs have a decreased diameter, are more vulnerable and therefore break easily. The hair loss pattern is classified according to Ludwig as shown in Figure 10.[16]

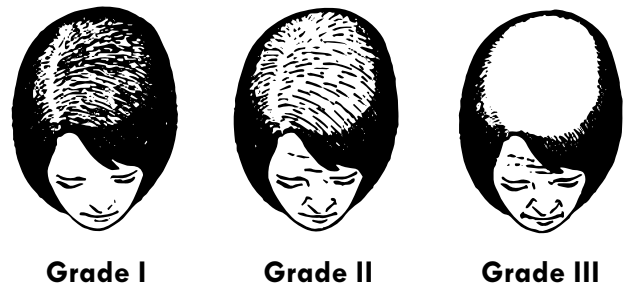


Figure 10: Female AGA classification according to Ludwig.[16] Figure from a previous publication.[50]

THERAPY

Drug treatment

Although drug therapy is limited, there are various possibilities. Medication for the treatment of patients with AGA can be divided into three groups.

Topical

Topical drugs can influence hair growth through a specific receptor in the hair follicle. An example is Minoxidil (6-piperidin-1-ylpyrimidine-2,4-diamine 3-oxide), a Potassium (K+) channel opener (PCO). Minoxidil finally stimulates the DNA synthesis in the hair follicle, which promotes hair growth.[17] Although hair regrowth is proven, the limitation of the compound is that the hair follicle should still be in the anagen phase and the maximum result one may expect is stabilization of the hair loss process.[5]

Systemic

Anti-androgens are drugs that block the androgen receptor or influence the androgen hormone system. For men, finasteride and dutasteride are available. Finasteride was originally prescribed for benign prostatic hypertrophy and halts 5-α-reductase type II (present in the hair follicles) by a more than 100-fold selectivity as compared with 5-α-reductase type I and blocks the peripheral conversion of testosterone into dihydrotestosterone. Finasteride is especially effective in the early stages of AGA in men. The effect of finasteride was demonstrated in three trials with 1,879 men aged 18-40 years with mild to moderate, but not complete hair loss on the scalp and hair loss on the front/middle part of the scalp.[18,19,20] There was no effect in post-menopausal women with AGA who were treated for 12 months with finasteride 1 mg 1 dd.[21]

However, finasteride doses of 2.5 or 5 mg 1 dd did prove to be effective in women.[22,23] Dutasteride, another 5-α-reductase blocker that blocks both types I and II 5-α-reductase[24] appeared to be more effective than finasteride.[25] The anti-androgenic drug cyproterone acetate, which is exclusively used in women, blocks the androgen receptor and inhibits the effect of dihydrotestosterone. Other drugs that influence the androgen receptor are the progesterone products, such as 11-α-hydroxyprogesterone. Cimetidine and spironolactone were used in the past, but currently have no clinical value.[26,27]

Formulated products which are sold mainly at drugstores and pharmacies over the counter, are usually a combination of unspecified substances or natural supplements. The effectiveness has not been scientifically proved and there is a lack of knowledge on the ingredients. Therefore, such products cannot be recommended.

In the European Dermatology Forum (EDF) guidelines only minoxidil, finasteride and dutasteride (only in men) are recommended for the treatment of AGA.[28] A recent meta-analysis in which 23 out of the 45 scientific articles were selected strongly indicated that minoxidil, finasteride, and low-level laser light therapy were effective for promoting hair growth in men with AGA and that minoxidil was effective in women with androgenetic alopecia.[29] Although the use of low-level laser light system in AGA may be effective, the experience in daily practice is limited and more research is needed to evaluate its value in the treatment of AGA.

The disadvantage of all the treatments for AGA mentioned above is the fact that the effectiveness persists as long as the medication is used. Especially for Finasteride (and Dutasteride), the long-term safety effects of finasteride and dutasteride were reported in only one study.[30]

Surgery is favorable as a more permanent solution both from a difficult acceptance of lifetime drug treatment for AGA as well as the cost effectiveness points of view.

SURGERY

Surgery is the only possibility to achieve permanent hair growth. To date, surgery varies from single hair follicle transplants to full-thickness flaps. Full-thickness flaps like the "Juri" flaps are not performed frequently anymore because of the poor cosmetic results.[31] The success of hair transplantation is explained by the fact that the transplanted hair follicle has the same characteristics in the new site as those in the donor site. Even in the most advanced stages of AGA, a horseshoe-shaped rim of hair remains, which is insensitive to dihydrotestosterone both in men and women (Figures 9 & 10). Hair follicles that are removed from the donor area to a bald spot on the scalp of the same person will develop new hairs.[32] The cosmetic results depend not only on the type of grafts (single-hair grafts, partial follicular units or follicular units), the survival rate of the transplantation and the skill of the surgeon, but also on the number of grafts which can be transplanted.

There are different techniques of hair transplantation, all with their own advantages and disadvantages. The most common and well-known hair transplantation method is the so called "strip" method.[33] A strip of skin containing hair follicles is removed, cut into grafts and implanted in the recipient area. The donor area is stitched, which leaves a visible linear scar. Other methods were developed in the last decade. The most promising method was the Follicle Unit Extraction (FUE) method.[34] In this method, whole follicle units are extracted one by one and are implanted one by one back into the recipient area. The FUE method was a major step towards the perfection of hair transplantation. Although, the FUE method is more patient friendly and leaves only tiny scars at the donor site as compared with the strip method, which leaves visible linear scars, the major disadvantage of both methods is that the extracted hair follicles are removed and the source of potential grafts is exhausted in time. Hair transplantations with the described methods will always be seriously limited by the availability of donor hair follicles and results in decreased hair density because no hair re-growth occurs in the donor area.

Currently, multiplication of human hair follicles in vitro is not possible. In theory, significant parts of donor hair follicles could be preserved by partial follicular unit extraction. These parts should possess sufficient growth capacity to develop into new HFs in vitro as well as in vivo. This idea is not unrealistic and supported by different in vivo experiments.[35] Kim and Choi[35] reported that in humans, the proximal part (the part which is the nearest to the dermal papilla) of the hair follicle cannot regenerate into a differentiated hair follicle, but the distal part of the follicle may eventually result in a fully developed hair follicle.[36] Reynolds et al[37] reported that in humans, the sheath of the lower part of the hair follicle is capable to induce hair regeneration, in contrast to the dermal papillae. These apparently contradictory results indicated that both proximal and distal areas of the hair follicle may contain follicular stem cells that are able to induce hair growth.

Commo et al.[38] also reported that distinct areas in the hair follicle from skin biopsies were positive for CK19. The fact that these areas were also Bcl-2 positive and Bax negative was a strong indication for different follicular stem cell sites, that may induce hair growth. Positivity for CK19 and Bcl-2 corresponded to infrequent cell division in these areas as concluded from the absence of Ki-67 positive cells.[39] The fact that these cells were positive for Bcl-2 and CK 19, but were negative for Ki-67 and Bax was a strong indication that they represented follicular stem cells in the hair follicle.

Interest in stem cells has increased dramatically in the last decade, particularly with regards to their role in the rapidly expanding fields of cosmetic & regenerative medicine and tissue engineering.[40] In the field of hair restoration, follicular stem cells could be important to regenerate new hair follicles. The knowledge that major parts of the hair follicle contain follicular stem cells may offer the attractive possibility to divide a hair follicle into several pieces to create new hair follicles. However, in order to be able to understand and to guide this process, it is important to know the background and characteristics of (follicular) stem cells as well as their potential capacity to (re)generate hair.

Stem cells may be distinguished in embryonic and adult stem cells. These stem cells can be: Totipotent (a.k.a. omnipotent) stem cells are stem cells with the ability to develop into all types of living cells with the potential to generate whole new individuals. Pluripotent stem cells are the descendants of totipotent stem cells and may create all cell types except those belonging to extra embryonic tissue such as placenta. A pluripotent stem cell can differentiate into 3 germ layers i.e. endoderm, mesoderm and ectoderm. Multipotent stem cells can differentiate into pertinent types of cells, only those of within a certain dermal lineage. Oligopotent stem cells can differentiate into only a few cells of a particular tissue. Unipotent stem cells can produce only their own cell type, but have the property of self-renewal, which distinguishes them from non-stem cells.

Embryonic stem cells are pluripotent while postnatal i.e. adult stem cells, which are generally considered to be multipotent.

In the clinical setting, stem cells may be distinguished into autologous stem cells, which are stem cells from the own body, heterologous stem cells, which are stem cells derived from the same species (human), but from another person or (human) embryo and xenologous stem cells, which are stem cells derived from another species than human (pigs, rats, etc.).

It has been reported that the hairfollicle (HF) contains stem cells of different potency (pluri-, multi- and unipotent stem cells).[41,42,43] The challenge is to select the desired cell population and to control their differentiation. It is of importance to realize that 2D cultures of stem cells are of limited use when studying the mechanism of pathogenesis of diseases and the feasibility of a treatment. Therefore, as a proof of principle of the regenerative qualities of HFs, we focused our research on in vivo experiments i.e. placement of (partial) HFs in the skin of the patient. This 3D environment is the most suitable environment for regrowth of HFs, but recent work suggests that there is also perspective for in vitro stem cell research (reviewed in: de Groot et al. submitted to Anatomical Record). Although other factors play a role, the extra cellular matrix (ECM), including its topography, is crucial to mimic a stem cell niche in vitro and to drive stem cells towards formation of the tissue of interest. Technological developments have led to the investigation of biomaterials that closely resemble the native ECM.

The procurement of autologous somatic stem cells for human therapeutic purposes is still limited. In addition, somatic stem cell potency is restricted and are multipotent rather than pluripotent.[40] Reprogramming somatic cells into pluripotent stem cells, i.e. induced pluripotent stem cells (iPSC),by the forced expression of certain genes is being explored, but is controversial because iPSC are often tumorigenic and may initialize a T cell-dependent immune response in syngeneic recipients.[44,45]

For these reasons, the use of other types of autologous somatic stem cells such as bone marrow stem cells in a curative treatment for ischemic heart patients and cerebral infarction is currently under investigation and clinical trials and animal studies already show some promising results.[46,47] However, one of the risks is that multipotent stem cells may follow their innate biological inclination irrespective of the tissue or organ into which they have been grafted. This was demonstrated by the finding that autologous bone marrow stem cells can produce extracellular matrix after engraftment into the brain.[48,49]

In AGA-cell-based therapy, autologous multipotent stem cells from the HFs may thus provide an attractive therapeutic option because they can be easily harvested using minimally invasive techniques. Moreover, it may be possible that the relatively immune privileged tissue of the hair follicle, HF stem cells may also be promising candidates for allogeneic stem cell therapy.

For other future clinical applications, it would be ideal to have pluripotent or multipotent adult stem cells, which can regenerate a damaged or diseased organ or skin while minimizing the need for systemic immunosuppression. It is of interest that the HF also contains pluripotent stem cells, which will reduce ethical and regulatory issue.[40]

Therefore, given the novelty of this type of stem cell in the field of cell-based regenerative therapy and particularly in the field of skin regeneration, it is required to investigate HFSC characteristics while focusing on their proliferative and differentiation potential.

AIMS OF THE THESIS

The general aim of this thesis is:
To demonstrate that the population of follicular stem cells consists of pluripotent, multipotent, oligopotent and unipotent stem cells and that these stem cells may be stimulated to generate fully differentiated hairs. Therefore it would not be necessary to transplant the whole hair follicle to regenerate new hairs, but only (a part of) the follicular stem cells. In the investigations described in this thesis, we investigate the characteristics of different follicular stem cells and their potential applications.

The main objectives of this thesis were:

- **A.** To identify and determine the localization of the follicular stem cells in whole hair follicles, plucked hairs and hair follicular cell culture.
- **B.** To study the viability of hair transplantation grafts and hair follicular units.
- **C.** To explore the hair growth potential of partial follicular units containing follicular stem cells and their clinical applications and to evaluate the results.
- **D.** To study hair follicular stem cells and to explore their potential future applications in regenerative medicine.

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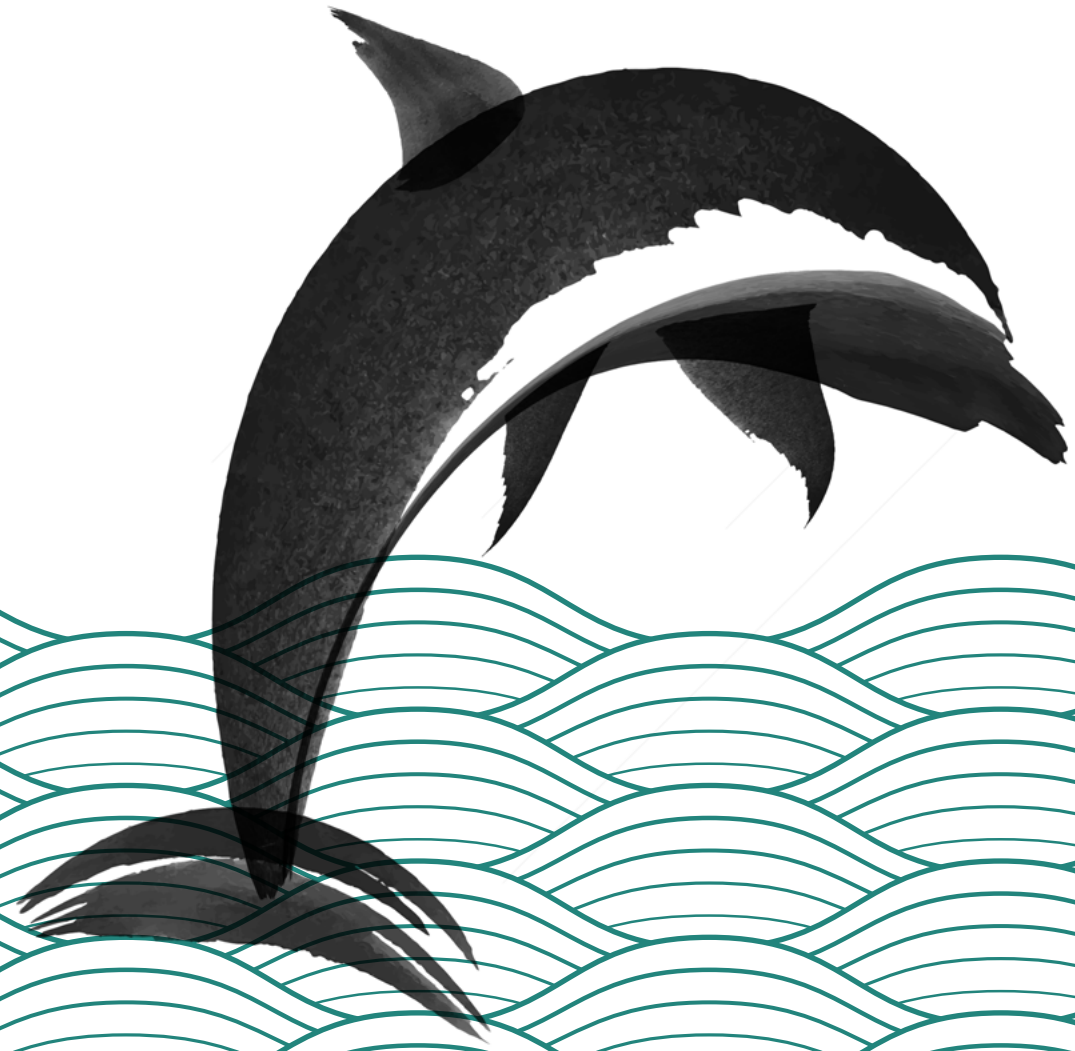

7

THE LOCALIZATION OF
THE FOLLICULAR STEM CELLS IN PLUCKED HAIR AND FOLLICULAR CELL CULTURE

2. HUMAN FOLLICULAR STEM CELLS:
THEIR PRESENCE IN PLUCKED HAIR AND FOLLICULAR CELL CULTURE

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"Human follicular stem cells: their presence in plucked hair and follicular cell culture." Br J Dermatol 150(5): 860-868.

SUMMARY

Background and objectives
A considerable portion of the hair follicle remains attached to plucked hair and can be used for follicle cell culture. In this study we have phenotyped these cells in an attempt to identify the stem cell fraction. Reports in the literature have indicated that this cell population may be positive for cytokeratin (CK) 19. Because stem cells in general need to be protected from apoptosis, the presence of the apoptosis-suppressing Bcl-2 protein, together with the absence of the apoptosis-promoting Bax and the CK profile may be used as an indicator of the stem cell population in the hair follicle, and in cultures of hair follicle cells.

Methods
Hair follicles from skin biopsies and plucked hair were derived from the scalps of healthy volunteers. Follicular cells were cultured from the plucked hairs. These hair follicles, plucked hairs and cultured cells were examined for their CK profiles, which are indicative of the type of cell (basal/stem cells) and for their status with respect to the proliferation marker Ki-67, Bax and Bcl-2.

Results
We found co-expression for CK19 and Bcl-2, but not Bax in two distinct areas, localized in the upper and lower third of the follicle from both skin biopsies and plucked hairs, while proliferation markers were negative in these areas. CK19 and Bcl-2 were also co-expressed in combination in a fraction of the follicular cell culture. The skin basal cell marker CK14 could be found throughout the outer root sheath of the hair follicle from both skin biopsies and plucked hairs, as well as in the follicular cell culture.

Conclusions
Thus, CK19/Bcl-2-positive and Bax-negative cells can be obtained from cells derived from plucked hair and are retained in cultures made from these cells. If this phenotype represents follicular stem cells, our finding endorses the assumption that stem cells are located in the bulge area of the hair follicle, as we did not find them in or near the dermal papilla.

INTRODUCTION

Although a considerable portion of the hair follicle is attached to plucked hair, transplantation of a plucked hair does not result in normal hair growth,[1] in contrast to hair from micropunch grafts.[2] This can be explained by the fact that the structures of the hair follicle which are retained in the skin after the hair is plucked, are necessary for normal hair growth. However, when outer root sheath (ORS) cells derived from plucked hairs are cultured, they can develop into a differentiated epidermis,[3] suitable for use in skin grafts.[4] We therefore speculated that the plucked hairs themselves may contain epidermal stem cells.

When plucked hair is dissected, the various transverse sections have been shown to exhibit different proliferative and differentiative characteristics.[5] It has been suggested that adult human follicular stem cells from terminal hairs are situated in the bulge area of the follicle. These cells have been suggested to exhibit various specific biochemical properties. For example they have epidermal growth factor (EGF) receptors, show alpha2beta1- and alfa3beta1-integrin expression,[6] high levels of alpha6-integrins and low levels of the proliferation marker 10G7,[7] and stain positively for platelet-derived growth factor (PDGF)-A/PDGF-B ligand chains. They do not contain nectadrin, or heat-stable antigen (CD24), a glycoprotein thought to be involved in cell–cell adhesion and signalling, which is also expressed in the outer epithelial sheath of human hair follicles and in glabrous epidermis.[8,9] However the CK apoptosis resistance profiles seem to have become key indicators of a stem cell phenotype.

During development into a terminal hair, the CK profile of the follicular keratinocyte changes. It has been proposed that CK19 is an indicator of the stem cell population.[10,11,12,13] CK19 is present in immature epithelial progenitor cells,[14] but in the hair follicle it is specific for follicular stem cells.[10,12] In adult hair follicles, CK19 can be found in the outermost cells of the ORS at the isthmus and in some cells of the lower ORS.10 It is proposed that the actual follicular stem cells are CK19-positive and lack connexin (Cx)43, a specific differentiation marker for a gap junction protein.

A balance between cell proliferation, differentiation and apoptosis is essential for hair growth,[15,16] while stem cells must be protected against apoptosis. This protection is achieved by proteins such as Bcl-2, while Bax, a conserved homologue that heterodimerizes with Bcl-2 promotes cell death.[17] The characteristics of the CK profile, in particular the expression of CK19, but also CK5 and CK14 as basal cell markers,[12] together with the expression of Bcl-2 and absence of Bax expression, may therefore be used as indicators of the stem cells in the hair follicle.

The aim of this study was to localize the follicular stem cells in the hair follicle from skin biopsies and to characterize them on the basis of their CK phenotype, presence of Bcl-2, and absence of Bax. Furthermore, we wished to determine whether these cells are present in plucked hair and preserved in cell cultures derived therefrom.

MATERIALS AND METHODS

Skin biopsies
Five healthy volunteers, three males and two females between the ages of 28 and 53 years (mean age 37 years) donated skin biopsies.

Biopsies
Four 3-mm punch biopsies were obtained from the occipital area of the scalp after local anaesthesia with lidocaine 2%. The tissue samples were immediately frozen in liquid nitrogen and stored at –80°C until use. Samples were then cut into 5-µm thick sections in a vertical direction and carefully placed on Superfrost plus slides (Menzel-Glaser, Braunschweig, Germany).

Plucked hair
Plucked hairs were obtained by removing the hairs with a depilation forceps from the occipital area of the scalp. Hair follicles in the anagen phase were selected under a dissection microscope, and embedded in Tissue Tek (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands), and directly cut into 5-µm sections as described above.

Cell cultures
Plucked hairs were placed in a Petri dish with Defined Serum-Free Keratinocyte Growth Medium (dSFK; Life Technologies B.V. Breda, The Netherlands). The nonviable, keratinised part of the hair follicle was removed under a dissection microscope. The hair follicles were subsequently put in a sterile culture disk and incubated in dSFK containing 20 U/ml dispase (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 30 minutes in a CO_2 incubator at 37°C. After this preincubation step, the hair follicles were transferred to a 24-well culture disk containing dSFK with 500 µg/ml penicillin (Life Technologies B.V. Breda, The Netherlands) and 0.25 µg/ml streptomycin (Life Technologies B.V. Breda, The Netherlands), and placed for 14 days at 31°C (as suggested previously by Imai et al.[18]) in a humidified atmosphere containing 5% CO_2. The culture medium was carefully removed every three days and replaced by fresh culture medium. The cells remained attached to the hair follicles during this culture period. After 14 days the culture medium was removed and replaced by a 0.5 mg/ml trypsin, 0.2 mg/ml EDTA (ethylene diaminetetraacetic acid) solution (Life Technologies B.V. Breda, The Netherlands), and incubated for 5 minutes at 37°C in this medium. After this incubation period clusters of cells were released from the hair follicles. These were harvested by centrifugation at 300 g at 4°C for 5 minutes in a Eppendorf 5804R Centrifuge (VWR International, The Netherlands). Subsequently the trypsin/EDTA medium was removed and replaced by culture medium. Cytospins were made from these cells in the Cytospin 3 (SHANDON, Zeist, The Netherlands) by centrifugation at 600 rpm for 5 minutes (300G).

These cells where immunostained as described below. Because the number of cells obtained in these cultured was very limited, reliable quantification of the immuno-positive cells could not be performed. Therefore, wherever possible, an indication of the percentage of positive cells is provided.

Antibody/Clone	Antibody specificity	Dilution	Isotype Monoclonals	Source/Reference
Basal cell markers		*(UD: undiluted culture supernatant)*		
LL002	CK14	UD	Mouse IgG3	[40]
RCK107	CK14	UD	Mouse IgG1	[41]
RCK108	CK19	1:5	Mouse IgG1	[42]
LP2K	CK19	UD	Mouse IgG2b	[43]
Proliferation markers				
LL002	CK16	1:10	Mouse IgG1	[41]
LL025	Ki-67	1:25	Mouse IgG1	Immunotech-Coulter, Marseille, France
MIB-1	Ki-67	1:100	Rabbit IgG	Dako A/S, Glostrup, Denmark
Apoptosis markers				
N-19 polyclonal	Bcl2	1:200	Rabbit IgG	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
124	Bcl-2	1:80	Mouse IgG1	Dako A/S Glostrup, Denmark
PC66	Bax	1:750	Rabbit IgG	Oncogene Research Products, Cambridge, MA, USA

Table 1: Table of markers and protocol used for staining of the tissue, plucked hairs and cell culture.

Immunocytochemistry and antibodies (Table 1)
The sections and cytospins were dried at room temperature for at least 1h, fixed in acetone (at –20°C) and processed for immunohistochemical staining.

For the single immunostaining procedure, the slides were incubated overnight at 4°C with the primary antibodies. All incubations were carried out in phosphate-buffered saline (PBS) pH 7.4 at the appropriate dilution (Table 1). The following day the slides were washed with PBS and incubated at room temperature for 30 min with the appropriate immunofluorescent-labeled secondary antibody (Table 1). After extensive washing, the slides were mounted with a 4´,6- diamidino-2-phenylindole (DAPI)-containing mounting agent (Vector Laboratories Inc.' Burlingame, CA, USA) and stored at –20°C.

For double-immunostaining, the primary antibodies were selected on the basis of their isotypes, or a combination of monoclonal (mouse source) and polyclonal (rabbit source) antibody was applied. The first primary antibody was incubated overnight at 40°C and the next day, after washing, the second primary antibody was incubated at room temperature for 2 h. After extensive washing in PBS, a mix of appropriate secondary antibodies (obtained from Dako A/S, Glostrup, Denmark or ITK Diagnostics, Uithoorn, The Netherlands) was applied and incubated for 30 min at room temperature. Subsequently the slides were washed in PBS, mounted as described above, and stored at –20°C.

Amplification of signal was achieved by incubation with biotinylated goat-anti-rabbit (BIO-GAR) or biotinylated goat-anti-mouse (BIO-GAM) and Avidin-Biotin complex (Vectastain ABC kit, Vector Laboratories Inc.' Burlingame, CA, USA) after incubation with the primary antibody. Detection of peroxidase activity and simultaneous signal amplification was achieved by incubation with tetramethylrhodamine isothiocyanate (TRITC)-labeled tyramide or fluorescein isothiocyanate (FITC)-labeled tyramide.[19]

RESULTS

Haematoxylin and eosin (HE) staining of skin biopsies and plucked hair

Comparison of the hair follicles derived from skin biopsies and from plucked hairs using light microscopy revealed that most of the epithelial structures from the hair follicle remain attached to the plucked hair (Fig. 1).

Figure 1: Haematoxylin and eosin staining of plucked hair. Most epithelial structures from the hair follicle remain attached to the plucked hair. Adjacent to the longitudinal section of the plucked hair (original magnification x10) the respective areas are shown at a higher magnification (x40).

Basal cell makers: CK14 and CK19

Cells positive for the basal cell markers CK14 (RCK107, LL002) were found in the basal cells throughout the whole length of the ORS of the hair follicle and in the basal cell layer of stratified epithelium of the epidermis, both in the skin biopsy samples and in the plucked hairs.

In the hair follicles from the skin biopsy, the basal keratinocyte cell marker CK19 (RCK108, LP2K) was found in the most peripheral cell layer in two distinct areas in the upper (Fig. 2A) and lower third (Fig. 2B) of the ORS, but not in the epidermis. In the plucked hair, the same regions were found to be positive for CK19 (Results not shown). In follicular cell cultures approximately one fifth of the cells were CK19 positive. These CK19-positive cells were mostly found as cell clusters, as shown in Fig. 3B.

A complete overlap of CK19 (RCK108) and CK14 (LL002) was found in the biopsy samples and in plucked hair, and in approximately 40% of the cultured cells (not shown).

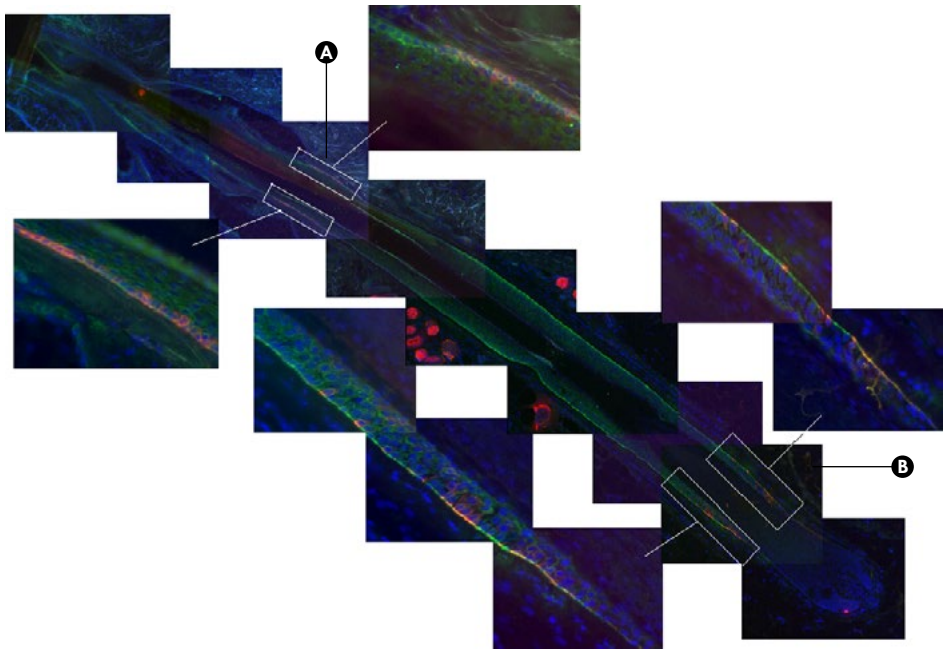

Figure 2: Longitudinally sectioned hair follicle from skin biopsies (original magnification x10) double stained for CK19 in red and Bcl-2 in green. Higher magnifications (x40) of areas found to be positive for both markers are shown next to the respective areas of the hair follicle. Two areas were found to be positive for both markers; i.e. the upper third ***(A)*** *and lower third* ***(B)*** *of the hair follicle. 4',6-diamidino-2-phenylindole (DAPI) counterstaining of DNA (blue).magnification (x40).*

(Anti)-apoptosis markers: Bcl-2 and Bax

Bcl-2 (N-19) positive cells were found in the outermost cell layer throughout the whole length of the ORS of the hair follicle from skin biopsies, as well as in the basal layer of the epidermis. We also found Bcl-2 positive cells in the most peripheral cell layer of the whole length of the ORS of the plucked hairs. Bcl-2 positive cells were not found in the dermal papilla. Double staining for CK19 (LP2K) and Bcl-2 (N-19) revealed that CK19-positive cells were also positive for Bcl-2. However, not all Bcl-2-positive cells were positive for CK19 (Fig. 3A, B).

In the follicular cell cultures subjected to double-staining techniques, some cells stained positive for both Bcl-2 and CK19, while others were positive for either Bcl-2 or CK19 (Fig. 4A, B). Figure 3B shows a typical cluster of CK19 positive cells, found in the cytospins of the cell cultures.

Bax (PC66)-positive cells were present in the internal part of the ORS both in the hair follicles from skin biopsies and in the plucked hair. Bax-positive cells were found in the dermal papilla in the hair follicles from skin biopsies (Fig. 4A) and in the most proximal part of the plucked hair (Fig. 4B). Almost no overlap (<5%) between Bcl-2 (124) and Bax (PC66)-positive cells was found in either sample. However, in follicular cell culture there was more overlap (30–40%) between Bcl-2- and Bax-positive cells (results not shown).

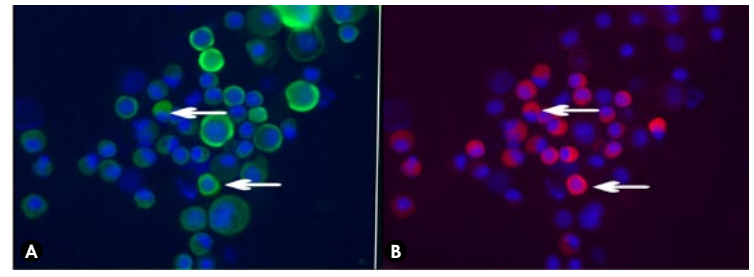

Figure 3: ***(A)*** *Bcl-2 (green) and* ***(B)*** *CK19 (red) staining of cells, derived from plucked hair and cultured for 14 days. Next to cells that are positive for both markers (arrows), some cells are only Bcl-2 or CK19 positive. 4',6-diamidino-2-phenylindole (DAPI) counterstaining of DNA (blue).*

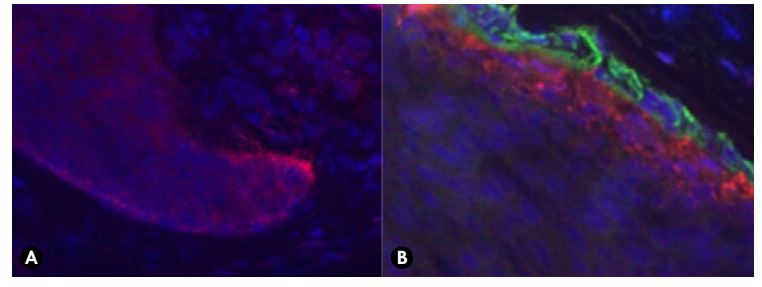

Figure 4: Details (original magnification x40) from a longitudinally sectioned dermal papilla ***(A)*** *and hair follicle* ***(B)*** *from skin biopsies double stained for Bax in red and Bcl-2 in green. 4',6-diamidino-2-phenylindole (DAPI) counterstaining of DNA (blue).*

(Hyper)Proliferation markers CK16 and Ki-67

CK16 (LL025)-positive cells were found in the distal internal part of the ORS of the hair follicles, both from skin biopsies and plucked hairs (Fig. 5). No CK16 staining was observed in the most proximal part of the hair follicles, either from skin biopsies or plucked hairs. CK16-positive cells were found in the internal part of the ORS, in contrast to CK19-positive cells whose position was more peripheral. There was no overlap between CK16 and CK19 staining in the hair follicles from skin biopsies, plucked hairs or follicular cell culture. There was an overlap in the immunostaining patterns of CK14 (LL002) and CK16 (LL025) in the ORS of the hair follicles from skin biopsies and in the plucked hair, where CK16-positive cells were located. In follicular cell culture there was virtually a total overlap between CK14- and CK16-positive cells (not shown).

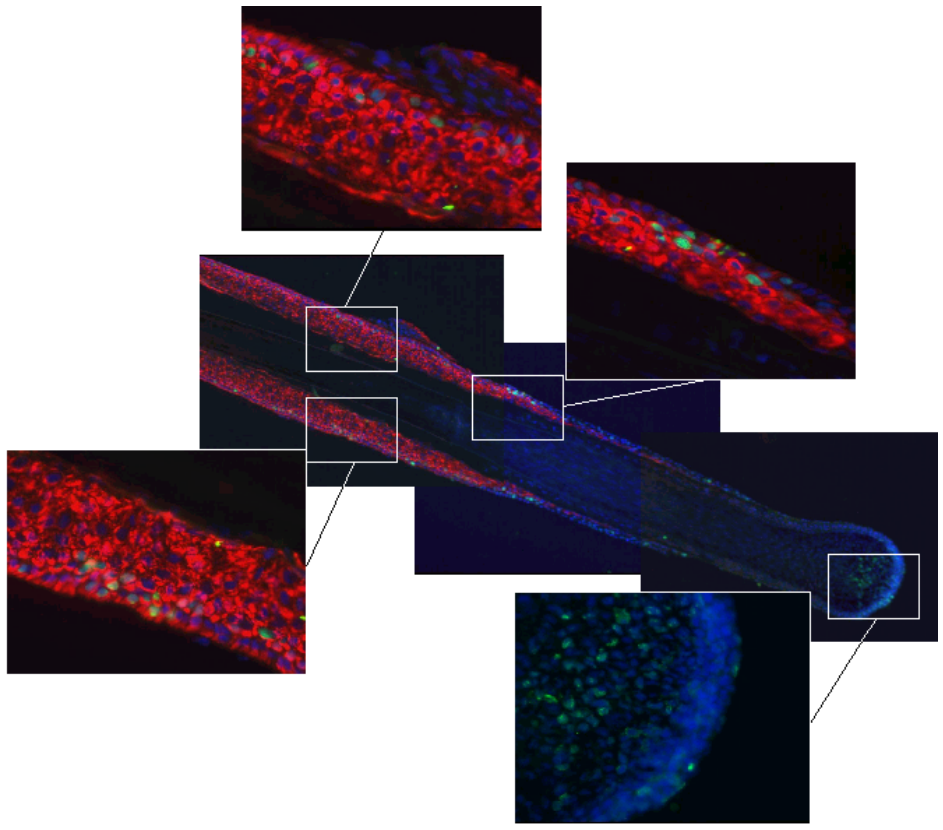

Figure 5: Longitudinally sectioned plucked hair (original magnification x10) double stained for CK16 in red and Ki-67 in green. Higher magnifications (x40) of areas found to be positive for both markers are shown next to the respective areas of the hair follicle. 4',6-diamidino-2-phenylindole (DAPI) counterstaining of DNA (blue).(DAPI) counterstaining of DNA (blue).

Ki-67-positive cells were sporadically found (Fig. 6). Their location tends to be more toward the internal part of the ORS, although some were found near the external part of the ORS near the CK19-positive cells. Ki-67-positive cells were also found in the dermal papilla (Fig. 5). Less than 1% of Ki-67-positive cells were found in follicular cell culture.

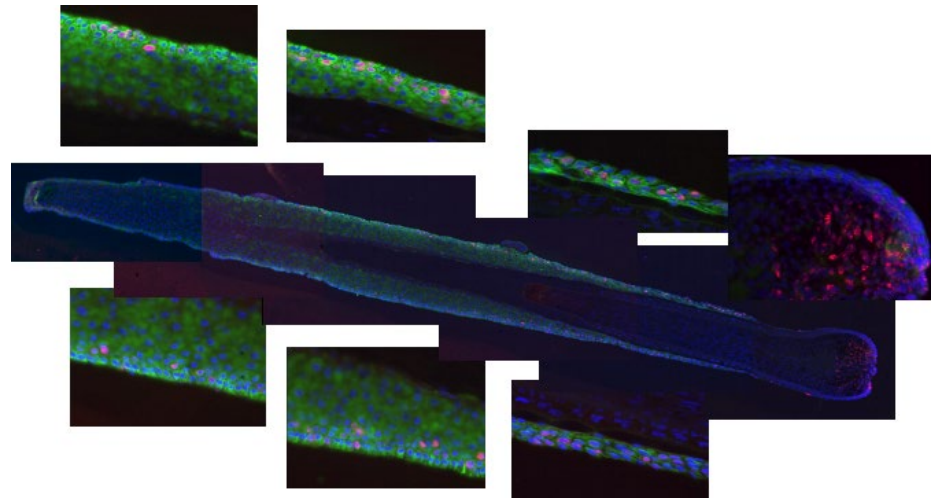

Figure 6: Longitudinally sectioned plucked hair (original magnification x10) double stained for Bcl-2 in green and Ki-67 in red. Higher magnifications (x40) of areas found to be positive for both markers are shown next to the respective areas of the hair follicle. 4',6-diamidino-2-phenylindole (DAPI) counterstaining of DNA (blue).

DISCUSSION

Although plucked hair seems to contain all of the epithelial structures which are present in the hair follicle derived from skin biopsies, the transplantation of plucked hair does not result in normal hair growth, in contrast to hair-containing punch grafts.[1] The fact that the structures of the hair follicle remaining in the skin can produce a normal hair, suggests that follicular stem cells are retained in the skin. However, when hair follicle preparations derived from skin biopsies are compared with preparations from plucked hair under a light microscope, it can be seen that the majority of the epithelial structures from the hair follicle remains attached to the plucked hair. The question thus arises whether or not follicular stem cells are extracted with the plucked hair, and whether the stromal tissue surrounding these epithelial structures is necessary for induction of hair growth. This follicular connective tissue has specific biochemical characteristics, such as the presence of PDGF-alpha and PDGF-beta receptors and versican,[20,21] that seem to play a role in (embryonic) hair follicle development and cycling.

We based our search for the follicular stem cell population in plucked hair and cultures thereof on stem cell characteristics proposed in the recent literature,[9] i.e. the expression of CK19 and Bcl-2, in combination with the absence (or low levels) of Bax expression,[22] and an extremely low proliferation frequency.[23] In 1996, Michel et al.[24] suggested that CK19-positive cells in hair follicles represent stem cells. Furthermore, stem cells in general express Bcl-2.[25,26] In normal skin, Bcl-2 is only expressed by a limited population of cells in the basal compartment, which can be regarded as the stem cell compartment.[27]

An important finding in this study is that phenotypical characteristics of the hair follicle from skin biopsies are preserved in plucked hair, including the expression of CK19[10] and Bcl-2[16] in cells from two areas of the upper and lower third of the follicle. Positivity for CK19 and Bcl-2 corresponds to infrequent cell division in these areas, as concluded from the absence of Ki-67 staining. The fact that these cells are positive for Bcl-2 and CK 19, but Ki-67 and Bax negative, is a strong indication that they represent stem cells in the hair follicle. Our results also indicate that transient amplifying cells in the epidermis, which are expected to be Ki-67 positive, are largely Bcl-2 negative. All the markers found in the hair follicle from skin biopsies were also preserved in the follicular cell culture derived from plucked hair, including CK19 and Bcl-2, and the basal cell marker CK14. We therefore conclude that viable follicular stem cells can be obtained from plucked hairs. The low frequency of Ki-67 positive cells indicates that differentiation may have occurred in these cell cultures, which can also be concluded from the CK10 staining in a proportion of the cells (unpublished observation).

The observation that two distinct areas in the hair follicle from skin biopsies are positive for CK19 was also found by Commo et al.[13] The fact that these areas are also Bcl-2 positive, and Bax negative, is a strong indication for two stem-cell sites. The question therefore arises whether or not both areas are necessary for hair growth induction. In humans, Kim et al.[28] found that the proximal part of the hair follicle cannot regenerate into a differentiated hair follicle, but the distal part of the follicle can, eventually resulting in a fully developed hair follicle.[29] Reynolds et al[30] found that, although the dermal papillae of humans cannot induce new hair growth, the sheath of the lower part of the hair follicle can. These apparently contradictory results indicate that both proximal and distal areas of the hair follicle can induce hair growth, which agrees with our finding of two stem cell locations.

In addition to these two areas, the dermal papilla has also been ascribed a key role in hair growth. In rats, cultured dermal papilla cells from whisker hairs can generate a fully differentiated hair follicle,[31] suggesting that follicular stem cells are located in or near the dermal papilla. The cells in this region that are held responsible for hair growth are the germ cells. Recently a hypothesis of hair cycling was proposed that involves participation of these germ cells next to the bulg region stem-cells.[32] In our study, the dermal papilla in the hair follicle of the skin biopsy did not contain Bcl-2. In contrast other studies have found Bcl-2 positivity in the dermal papilla,[17,33] but the tissues used in these studies were either embryonic or derived from non-melanoma skin cancers. The apparent discrepancy in findings could be due to the fact that, although Bcl-2 is normally found in the basal compartment of normal skin, there is an expansion of this Bcl-2 positive cell population under pathological circumstances such as basal cell carcinoma (BCC).[27,34]

In our study we found that the dermal papilla is positive for Ki-67 and Bax. This immunophenotype, in combination with the Bcl-2 negativity, is not in agreement with the characteristic definition of a follicular stem cell population, but rather supports the findings of others that follicular stem cells are not present in the dermal papilla.[16,35] However, taken all these findings together suggests that hair growth involves the co-operation of various stem cell regions.

The inability to successfully transplant plucked hairs containing the two stem cell regions may be due to the lack of appropriate conditions in the stromal tissue of the recipient areas. A similar lack of growth has been observed in case of transplanted epithelial structures derived from BCC which could not be induced to proliferate without the transplantation of additional stromal cell.[36] This comparison is justifiable since BCC are most likely derived from follicular germinative cells.[37,38] Another indication of the importance of stromal cells for the growth of keratinocytes is the observation that keratinocyte cultures may require a fibroblast feeder layer.[3]

Unsuccessful regeneration after implantation of plucked hairs may also be caused by the inflammatory response of the receptor area to the plucked hairs, which may result in the destruction of the follicular stem cells. The connective tissue surrounding these epithelial structures in micropunch grafts may be crucial for protection against inflammation, and thus a successful transplantation. Therefore, the importance of the connective tissue of the hair follicle cannot be ignored.[1,39] Follow-up studies will focus on the role of components of the connective tissue and the extracellular matrix surrounding the epithelial portion of the hair follicle.

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THE VIABILITY OF
HAIR TRANSPLANTATION GRAFTS AND FOLLICULAR UNITS

3.1 THE TYROSINE PHOSPHATASE INHIBITOR BIS(MALTOLATO)OXOVANADIUM ATTENUATES MYOCARDIAL REPERFUSION INJURY BY OPENING ATP-SENSITIVE POTASSIUM CHANNELS

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Liem, D. A., C. C. Gho, B. C. Gho, S. Kazim, O. C. Manintveld, P. D. Verdouw and D. J. Duncker (2004). "The tyrosine phosphatase inhibitor bis(maltolato)oxovanadium attenuates myocardial reperfusion injury by opening ATP-sensitive potassium channels." J Pharmacol Exp Ther 309(3): 1256-1262.

ABSTRACT

Vanadate has been shown to inhibit tyrosine phosphatase, leading to an increased tyrosine phosphorylation state. The latter has been demonstrated to be involved in the signal transduction pathway of ischemic preconditioning, the most potent endogenous mechanism to limit myocardial infarct size. Furthermore, there is evidence that phosphatase inhibition may be cardioprotective when given late after the onset of ischemia, but the mechanism of protection is unknown. We tested the hypothesis that the organic vanadate compound bis(maltolato) oxovanadium (BMOV) limits myocardial infarct size by attenuating reperfusion injury and investigated the underlying mechanism. Myocardial infarction was produced in 112 anesthetized rats by a 60-min coronary artery occlusion, and infarct size was determined histochemically after 180 min of reperfusion. Intravenous infusion of BMOV in doses of 3.3, 7.5, and 15 mg/kg i.v. decreased infarct size dose-dependently from 70 ± 2% of the area at risk in vehicle-treated rats down to 41 ± 5% ($P < 0.05$ versus control), when administered before occlusion. Administration of the low dose just before reperfusion was ineffective, but administration of the higher doses was equally cardioprotective as compared with administration before occlusion. The cardioprotection by BMOV was abolished by the tyrosine kinase inhibitor genistein and by the ATP-sensitive potassium (K^+_{ATP}) channel blocker glibenclamide but was not affected by the ganglion blocker hexamethonium. We conclude that BMOV afforded significant cardioprotection principally by limiting reperfusion injury. The mode of action appears to be by opening of cardiac K^+_{ATP} channels via increased tyrosine phosphorylation.

An increase in tyrosine residue phosphorylation via increased tyrosine kinase activity has been implicated in the signal transduction pathway of cardioprotection by ischemic preconditioning,[1,2,3] which is the most potent endogenous mechanism to limit myocardial infarct size. There is evidence that increased tyrosine residue phosphorylation, produced by a shift in the balance between tyrosine kinase and tyrosine phosphatase, increases white blood cell survival by inhibiting apoptosis.[4,5,6] Vanadate enhances tyrosine residue phosphorylation by inhibition of tyrosine phosphatase,[7,8] suggesting that vanadate may be of therapeutic benefit in myocardial infarction, which may involve both apoptosis and necrosis.[9,10] In support of this concept, Armstrong et al.[11] reported that serine threonine phosphatase inhibitors are highly effective in protecting isolated cardiomyocytes subjected to ischemia (without reperfusion), even when administered late (75 min) after onset of ischemia, suggesting that vanadate may not require administration before the onset of ischemia and might also act against reperfusion injury. However, to date, in vivo studies on the cardioprotective effects of tyrosine phosphatase inhibitors are lacking.

The mechanism by which tyrosine phosphatase inhibitors exert their cardioprotective effects is incompletely understood. However, since K^+_{ATP} channels have been reported to be downstream targets of tyrosine kinase in the signaling pathway of ischemic preconditioning,[1,3] we hypothesized that K^+_{ATP} channels contribute to the cardioprotection by vanadate. Finally, several studies, including from our own laboratory, have reported that a brief period of ischemia[12] or local intra-arterial infusion of adenosine[13] and bradykinin[14] in remote organs such as small intestine and kidneys can protect the myocardium by stimulation of afferent nerves in the remote ischemic organ that results in activation of a neurogenic pathway.[12,13] We hypothesized that activation of this neurogenic pathway (which implies that compounds are not required to reach the area at risk) might also contribute to the cardioprotection by vanadate administered intravenously just before reperfusion.

In view of these considerations, the present study was designed to investigate 1) whether pretreatment with bis(maltolato)-oxovanadium (BMOV) is cardioprotective; 2) whether BMOV treatment after the onset of occlusion, but just before reperfusion is still cardioprotective; and 3) the mechanism of protection by BMOV, including the involvement of K^+_{ATP} channel opening and a neurogenic pathway. All studies were performed in anesthetized open-chest rats subjected to a 60-min coronary artery occlusion.

MATERIALS AND METHODS

Guidelines for Animal Research

Experiments were performed in ad libitum-fed male Wistar rats (300 g) in accordance with the Guide for the Care and Use of Laboratory Animals *(National Institutes of Health publication no. 86-23, revised 1996)* and with approval of the Animal Care Committee of the University.

Surgical and Experimental Procedures

Pentobarbital-anesthetized (60 mg/kg) rats were intubated for positive pressure ventilation (Servo ventilator) with oxygen-enriched room air.[13,15] Through the carotid artery, a PE-50 catheter was positioned in the thoracic aorta for measurement of arterial blood pressure and heart rate. In the inferior caval vein, a PE-50 catheter was placed for infusion of physiological saline to maintain fluid balance. Following thoracotomy, via the left third intercostal space, the pericardium was opened, and a silk 6-0 suture was looped under the left anterior descending coronary artery for later coronary artery occlusion (CAO). A catheter was positioned in the abdominal cavity to allow intraperitoneal administration of pentobarbital for maintenance of anesthesia. Rectal temperature was continuously measured and maintained at 36.5° to 37.5°C.[16] To prevent local heat loss from the thorax, the thoracotomy site was covered with aluminum foil. After completion of surgery, a 30-min stabilization period was allowed before experimental protocols were carried out.

All rats were subjected to a 60-min CAO followed by 180 min of reperfusion. At the end of reperfusion, the left anterior descending coronary artery was re-occluded and the area at risk determined with negative trypan blue staining, after which the heart was excised and infarct size determined with negative nitroblue tetrazolium staining.[12, 13,15,16]

Rats that fibrillated were allowed to complete the protocol, provided that conversion to normal sinus rhythm occurred spontaneously within 1 min or that defibrillation by gently thumping on the thorax or with a 9V battery was successful within 2 min after onset of fibrillation. Occlusion and reperfusion were visually verified.[15]

Effect of BMOV on Infarct Size

To determine whether BMOV had any effect on infarct size 3 doses of BMOV (3.3, 7.5, and 15 mg/kg) or its vehicle [up to 2.5 ml of phosphate-buffered saline (PBS)] were administered over 10 min, starting 20 min before the 60-min CAO. We subsequently investigated whether attenuation of reperfusion injury contributed to the limitation of infarct size. Since the former experiments established a dose-dependent limitation of infarct size by BMOV, the same doses of BMOV or its vehicle were again administered over 10 min but now starting 10 min before reperfusion.

Mode of Action of BMOV

To establish whether the limitation of infarct size/reperfusion injury by BMOV required an increased state of tyrosine phosphorylation during reperfusion, we investigated whether the cardioprotection by BMOV, in a dose of 7.5 mg/kg i.v. administered either before occlusion or before reperfusion, was affected by the presence of the tyrosine kinase inhibitor genistein.[3,17] Genistein was administered intravenously in doses of either 5 or 10 mg/kg over 5 min, starting 15 min before reperfusion. To investigate the involvement of K^+_{ATP} channel opening in the protection by BMOV, we determined whether the K^+_{ATP} channel blocker glibenclamide affected the cardioprotection by BMOV (7.5 mg/kg) administered before reperfusion. Glibenclamide was administered in two doses of 3 mg/kg each and infused over a 5-min period, the first infusion starting 20 min before occlusion (to ensure sufficient incubation time for glibenclamide in the area at risk)[18] and the second infusion starting at 45 min after the onset of occlusion.

The involvement of indirect protection of the heart by stimulation of afferent nerves in a remote organ and subsequent activation of a neurogenic pathway was investigated by studying the effect of intravenous BMOV (7.5 mg/kg before reperfusion) in the presence of the ganglion blocker hexamethonium (20 mg/kg i.v.) administered over 15 min starting 35 min after the onset of occlusion.

Materials

BMOV (GHO-1; GHO-Pharma, Maastricht, The Netherlands) was dissolved in 1 ml (3.3 and 7.5 mg/kg) or 2.5 ml (15 mg/kg) of PBS (modified Sörensen). Genistein (5 and 10 mg/kg, Sigma-Aldrich, St. Louis, MO) was dissolved in 0.3 ml of 95% ethanol and alkamuls EL-620 (Rhodia, Lyon, France) to which 0.3 ml of physiologic saline was added. Glibenclamide (6 mg/kg, Sigma-Aldrich) was dissolved in 1 ml of deionized H2O at a pH of 10. Hexamethonium (20 mg/kg, Sigma-Aldrich) was dissolved in 1 ml of physiologic saline. Fresh drug solutions were prepared each day.

Data Analysis and Presentation

Infarct size was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. The importance of timing of administration of BMOV (preischemia versus prereperfusion) was analyzed by using two-way (timing dose) ANOVA. Hemodynamic variables were compared by two-way ANOVA for repeated measures followed by paired or unpaired Student's t testing. Statistical significance was accepted when $P < 0.05$. Data are presented as mean ± S.E.M.

RESULTS

Exclusion Criteria

Of the 128 rats that entered the study, 13 rats were excluded because of sustained ventricular fibrillation during coronary artery occlusion (no more than three rats in one group), and three rats were excluded because the area at risk comprised less than 10% of the left ventricular mass.

Effect of BMOV on Infarct Size

There were no differences ($P = 0.22$) between the areas at risk of the various experimental groups ($40 \pm 1\%$, $n = 112$). Infarct size, which was $70 \pm 2\%$ in vehicle-treated rats, was limited in a dosedependent manner to $41 \pm 5\%$ by administration of BMOV before the 60-min CAO ($P < 0.05$; Fig. 1, left). When administered just before reperfusion, BMOV in the dose of 3.3 mg/kg was ineffective, but the doses of 7.5 and 15 mg/kg were equally cardioprotective (both $P > 0.30$) as the corresponding doses administered before the 60-min CAO (Fig. 1, right).

Mode of Action of BMOV

The cardioprotection by BMOV in a dose of 7.5 mg/kg was abolished when rats were treated with genistein, independent of whether BMOV was administered before the 60-min CAO (Fig. 2, left) or just before the 180-min reperfusion period (Fig. 2, right). The inhibition by genistein of the infarct size limitation by BMOV occurred in a dose-dependent fashion because 5 mg/kg only partly blocked the protection by BMOV administered just before reperfusion [infarct size $60 \pm 4\%$ ($n = 5$; data not shown in Fig. 2); $P < 0.05$ versus both BMOV-treated ($44 \pm 3\%$) and PBS-treated ($70 \pm 2\%$) rats]. The cardioprotection by BMOV, administered before reperfusion, was also abolished by glibenclamide but not by hexamethonium (Fig. 3). Genistein (10 mg/kg; Fig. 2), glibenclamide (Fig. 3), and hexamethonium (Fig. 3) had no effect on infarct per se, which is in agreement with previous observations.[3,12,17,19]

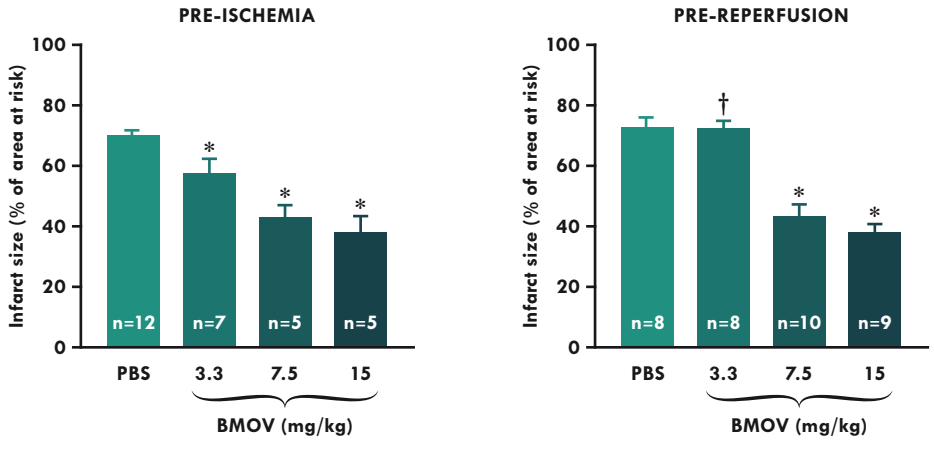

*Figure 1: Effects of BMOV administered before ischemia (left) or reperfusion (right) on myocardial infarct size produced by 60-min CAO. *, $P < 0.05$ versus corresponding vehicle (PBS); †, $P < 0.05$ BMOV prereperfusion versus BMOV pre-CAO.*

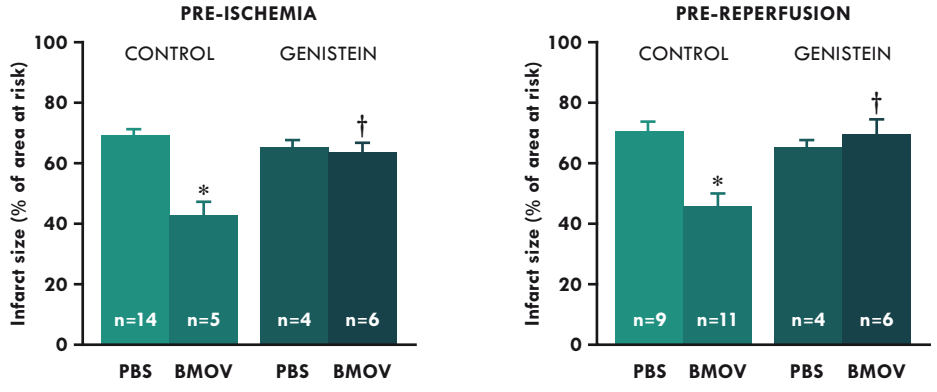

*Figure 2: Effects of the tyrosine-kinase inhibitor genistein (10 mg/kg) administered i.v. 15 min before reperfusion on the cardioprotection by intravenous administration of 7.5 mg/kg BMOV administered either before ischemia (left) or before reperfusion (right). *, $P < 0.05$ versus corresponding vehicle(PBS); †, $P < 0.05$ versus corresponding control BMOV.*

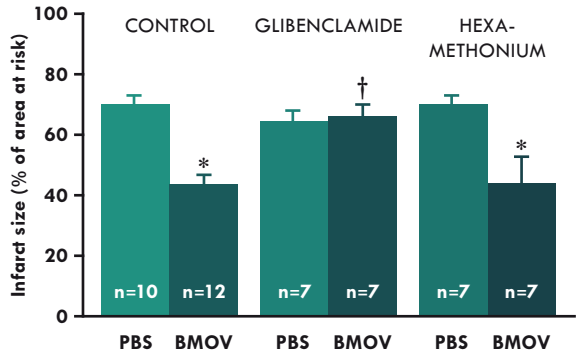

*Figure 3: Effects of the K^+_{ATP} channel blocker glibenclamide (2 x 3 mg/kg i.v.) or the ganglion blocker hexamemethonium (20 mg/kg i.v.) on the cardioprotection by 7.5 mg/kg BMOV intravenously administered before reperfusion. *, $P < 0.05$ versus corresponding vehicle (PBS); †, $P < 0.05$ versus corresponding control BMOV.*

Hemodynamic Effects of BMOV

There were no differences in baseline values of heart rate ($P = 0.37$) and mean arterial blood pressure ($P = 0.25$) between any of the experimental groups (Table 1). In the PBS-treated groups, heart rate and mean aortic blood pressure remained virtually unchanged throughout the 60-min CAO. During the subsequent 180-min reperfusion period, blood pressure slightly decreased, and heart rate slightly increased. BMOV, administered either before occlusion or before reperfusion, produced a transient and dose-dependent increase in mean arterial pressure, which was accompanied by a decrease in heart rate.

Apart from these transient effects, the hemodynamic responses to occlusion and reperfusion were not different from those in the vehicle-treated animals.

Pretreatment with 10 mg/kg genistein markedly attenuated the pressor response induced by 7.5 mg/kg BMOV (14 ± 5 compared with 31 ± 4 mm Hg, $P < 0.05$; Table 1). In contrast, the BMOV-induced increase in blood pressure was not altered by pretreatment with either glibenclamide (25 ± 9 mm Hg) or hexamethonium (53 ± 12 mm Hg).

Infarct size limitation by preocclusion treatment with BMOV was not related to alterations in the product of heart rate and mean arterial blood pressure at the onset of occlusion (Fig. 4, left), which is in line with previous observations that infarct size is not correlated with oxygen demand at the onset of occlusion.[12,16,20,21] In addition, the cardioprotection by BMOV administered before reperfusion was also not correlated with the rate-pressure product at the onset of reperfusion (Fig. 4, right). Together, these findings indicate that the infarct size limitation by BMOV cannot be explained by a decrease in global left ventricular energy demands.

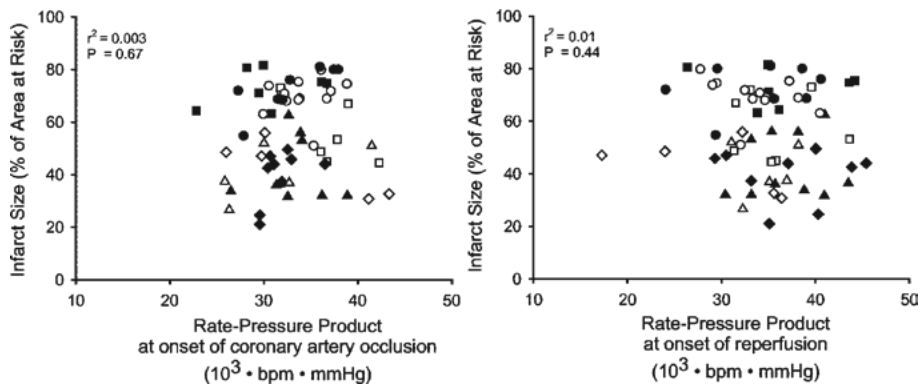

Figure 4: Lack of relation between the rate-pressure product at the onset of coronary artery occlusion (left) and reperfusion (right) and myocardial infarct size in rats receiving PBS or BMOV before occlusion (open symbols) or reperfusion (solid symbols). Data are presented as individual data points from animals presented in Fig. 1. Circles, PBS; squares, 3.3 mg/kg BMOV; upward triangles, 7.5 mg/kg BMOV; diamonds, 15 mg/kg BMOV.

TABLE 1
Heart rate and mean arterial blood pressure
Genistein was administered in a dose of 10 mg/kg (5 mg/kg not shown in Table 1). Data are mean ± S.E.M.

	Pre-Coronary Artery Occlusion (min)			Coronary Artery Occlusion (min)			Reperfusion (min)			
	−25	−10	−1	30	50	60	15	60	120	180
	min									
Pre-ischemia treatment with BMOV										
PBS pre-CAO ($n = 14$)										
HR	360 ± 8	357 ± 9	359 ± 8	368 ± 8	368 ± 8	379 ± 13	369 ± 9	379 ± 13	390 ± 11***	397 ± 9***
MAP	95 ± 3	100 ± 3	98 ± 2	95 ± 3	95 ± 3	92 ± 3	90 ± 4	86 ± 4	91 ± 4	91 ± 4
BMOV 3.3 pre-CAO ($n = 7$)										
HR	385 ± 12	297 ± 16*	367 ± 15	380 ± 14	378 ± 17	396 ± 15*,**	382 ± 18	396 ± 12*,**	398 ± 12*,**	407 ± 9*,**
MAP	99 ± 3	123 ± 4*	100 ± 3	92 ± 5	94 ± 4	93 ± 5	89 ± 5	88 ± 6	88 ± 6	87 ± 4
BMOV 7.5 pre-CAO ($n = 5$)										
HR	347 ± 11	251 ± 15*	310 ± 15	362 ± 7**,**	359 ± 12**,**	370 ± 16*,**	362 ± 14*,**	393 ± 17*,**	391 ± 10*,**	
MAP	91 ± 1	134 ± 5*	100 ± 5	101 ± 5	102 ± 3	91 ± 4	93 ± 1	80 ± 6**	80 ± 6**	
BMOV 15 pre-CAO ($n = 5$)										
HR	340 ± 9	298 ± 11*	334 ± 11	358 ± 9	360 ± 8	375 ± 6*,**	346 ± 14	409 ± 13*,**	400 ± 17*,**	
MAP	94 ± 3	138 ± 10*	102 ± 10	87 ± 12	84 ± 10**	85 ± 9*,**	84 ± 10**	84 ± 6**	72 ± 11*,**	
Pre-reperfusion treatment with BMOV										
PBS pre-REP ($n = 8$)										
HR	374 ± 15	342 ± 10*	338 ± 9*	344 ± 11*	341 ± 7*	355 ± 9*	341 ± 7*	373 ± 13**	383 ± 11**	
MAP	97 ± 2	99 ± 3	97 ± 3	95 ± 4	96 ± 5	92 ± 5	94 ± 4	89 ± 7	89 ± 3	
BMOV 3.3 pre-REP ($n = 8$)										
HR	387 ± 18	368 ± 10	362 ± 9	364 ± 5	364 ± 3	369 ± 7	371 ± 9	384 ± 10	405 ± 10**	
MAP	99 ± 5	95 ± 3	87 ± 4*	82 ± 5*	93 ± 4	82 ± 6*	94 ± 6	76 ± 5*	79 ± 6*	
BMOV 7.5 pre-REP ($n = 12$)										
HR	373 ± 7	340 ± 7*	337 ± 8*	345 ± 10*	340 ± 9*	343 ± 12*	285 ± 8**,***	369 ± 10**	371 ± 16**	
MAP	101 ± 4	97 ± 2	97 ± 2	95 ± 3	96 ± 3	84 ± 3*	127 ± 4**,***	84 ± 3*	84 ± 4*	
BMOV 15 pre-REP ($n = 9$)										
HR	367 ± 12	338 ± 6*	333 ± 3*	339 ± 5*	339 ± 5	344 ± 9*	274 ± 5**,***	357 ± 6**	379 ± 4**	
MAP	101 ± 4	97 ± 3	95 ± 2	97 ± 3	102 ± 2	93 ± 4*	135 ± 5**,***	83 ± 2**,**	88 ± 4**,**	
Mode of action of BMOV										
Genistein ($n = 4$)										
HR	366 ± 6	368 ± 10	369 ± 13	388 ± 18	388 ± 16	395 ± 12**	394 ± 13**	410 ± 12**	428 ± 16**	441 ± 10**
MAP	103 ± 6	115 ± 5	115 ± 4	88 ± 6**	91 ± 5**	88 ± 8**	85 ± 7**	88 ± 6**	84 ± 5**,**	84 ± 5**,**
BMOV 7.5 + genistein ($n = 6$)										
HR	351 ± 16	249 ± 9*	295 ± 10*	330 ± 10	300 ± 13*	357 ± 12**	355 ± 9**	345 ± 9**	332 ± 20	348 ± 15**
MAP	108 ± 2	161 ± 3*,**	115 ± 5	95 ± 5**	75 ± 8*,**	88 ± 4*,**	101 ± 5*,**	86 ± 5*,**	89 ± 7**	83 ± 9*,**
Genistein + BMOV 7.5 ($n = 6$)										
HR	375 ± 13	353 ± 14	333 ± 9*	369 ± 12	356 ± 18	358 ± 26	330 ± 6	395 ± 15**	402 ± 15**	387 ± 17**
MAP	101 ± 5	100 ± 3	106 ± 2	88 ± 4**	91 ± 8	83 ± 2**	105 ± 8*,**	83 ± 2**	94 ± 6	81 ± 5**
Glibenclamide ($n = 7$)										
HR	370 ± 11	351 ± 10*	377 ± 18	378 ± 12	365 ± 21	372 ± 12	380 ± 16	372 ± 19	366 ± 25	374 ± 45
MAP	103 ± 6	110 ± 8*	111 ± 8	96 ± 7	104 ± 6	98 ± 5**	106 ± 6**	95 ± 6**	91 ± 7**	86 ± 12*,**
Glibenclamide + BMOV 7.5 ($n = 7$)										
HR	336 ± 13	336 ± 10	341 ± 12	339 ± 10	343 ± 12	371 ± 13	327 ± 11	371 ± 13	372 ± 13	388 ± 15*,**
MAP	107 ± 6	122 ± 3*	125 ± 2*	104 ± 4	118 ± 3	97 ± 5**	106 ± 6	97 ± 5**	86 ± 8**	94 ± 6**
Hexamethonium ($n = 7$)										
HR	338 ± 10	338 ± 4	332 ± 4	340 ± 6	328 ± 7	339 ± 8	319 ± 8	339 ± 8	338 ± 8	337 ± 9
MAP	96 ± 4	100 ± 3	92 ± 5	92 ± 4	73 ± 3*,**	84 ± 5	77 ± 4*,**	86 ± 7	84 ± 8	82 ± 9
Hexamethonium + BMOV 7.5 ($n = 7$)										
HR	363 ± 16	365 ± 13	367 ± 13	365 ± 10	334 ± 10**	357 ± 18	298 ± 14*,**,***	351 ± 18	357 ± 14	378 ± 11
MAP	103 ± 6	101 ± 4	105 ± 3	100 ± 1	71 ± 4*,**	86 ± 3	124 ± 14*,***	83 ± 3*,**	84 ± 4**	87 ± 3

HR, heart rate (beats per minute); MAP, mean arterial blood pressure (mm Hg).
*$P < 0.05$ vs. −25 min precoronary artery occlusion (baseline); **$P < 0.05$ vs. −1 min precoronary artery occlusion; ***$P < 0.05$ 60 min vs. 50 min coronary artery occlusion.

DISCUSSION

The major findings in the present study in the in vivo rat heart are that 1) pretreatment with BMOV limited myocardial infarct size in a dose-dependent manner; 2) at sufficiently high doses, BMOV was equally cardioprotective when administered before reperfusion as compared with administration before coronary artery occlusion; 3) tyrosine kinase inhibition and K^+_{ATP} channel blockade abolished the cardioprotection by BMOV; and 4) ganglion blockade had no effect on BMOV's cardioprotection.

Importance of Dosage and Timing of Administration of BMOV. Previous in vitro studies have indicated that vanadate possesses cardioprotective properties.[22,23] For example, vanadate limited acidosis and lactate accumulation during global ischemia in isolated buffer-perfused rat hearts, although postischemic recovery of left ventricular-developed pressure was only minimally improved.[22] The results from this study are difficult to interpret, because vanadate was administered in a high dose of 40 M that produced marked cardiodepression at baseline, as reflected in the more than 50% reduction in left ventricular-developed pressure.[22] Furthermore, ischemia lasted up to 15 min, which in buffer-perfused rodent hearts may already result in significant necrosis.[24] As a consequence, without measurement of infarct size, no distinction can be made between a vanadate-induced attenuation of reversible contractile dysfunction (stunning) versus limitation of myocardial infarct size.

Takeuchi et al.[23] administered vanadate for a period of 3 to 4 weeks in a dose of 7.5 mg/kg p.o. per day to rabbits with pressure overload-induced left ventricular hypertrophy. When isolated hearts were then subjected to a 40-min period of global ischemia, vanadate-treated hearts showed reduced lactate release and improved postischemic recovery of left ventricular-developed pressure compared with vehicletreated hypertrophied hearts. However, since in these experiments the duration of ischemia exceeded 15 to 20 min, again no distinction can be made between a vanadate-induced attenuation of reversible contractile dysfunction (stunning) versus limitation of myocardial infarct size.[25]

The present study is the first to demonstrate that BMOV, in a dose-dependent manner, limits myocardial infarct size in vivo. At a low dose of 3.3 mg/kg, only preischemia treatment was effective in limiting infarct size, suggesting either that the compound exerts principally anti-ischemic actions or that tissue concentrations were too low at the onset of reperfusion. Rats lack a significant collateral circulation in the coronary vascular bed, so that administration of BMOV may not have reached the jeopardized myocardium in sufficient concentrations before the onset of reperfusion. However, at sufficiently high doses, BMOV was equally effective when administered before reperfusion as compared with administration before coronary artery occlusion. It could be argued that BMOV exerted at least part of its protective action by reducing ischemia injury during the last 10 min of the 60-min CAO. This is highly unlikely, in view of previous observations in our laboratory in the identical experimental model in which a 45-min CAO followed by 180 min of reperfusion already resulted in an infarct size of 61 ± 7%, which was not significantly different from the infarct size of 70 ± 2% produced by a 60-min CAO.[16] As a consequence, any limitation of ischemia damage by BMOV, administered after 50 min of CAO, cannot explain the BMOV-induced limitation of infarct size to 43 ± 5%. Taken together, these findings suggest that BMOV exerts its effects principally during reperfusion but that sufficiently high concentrations need to be present in the blood at the onset of reperfusion.

Mechanism of Cardioprotection by BMOV. The cardioprotective effect of vanadate in isolated buffer-perfused rat hearts has been proposed to be in part mediated by its apparent free radical scavenging properties. Thus, vanadate has been reported to inhibit the generation of superoxide, resulting in a blunting of the superoxide-induced loss of sarcolemmal Ca2 pump activity and Na-dependent Ca2 uptake in isolated rat hearts.[26] In contrast, under certain conditions in vitro, vanadate has been shown to be capable of generating free radicals.[27] However, this does not account for all its actions because other investigators failed to observe any action of reductants or antioxidants on vanadate-induced expression of actin and c-Ha-ras.[28] Importantly, the role of reactive oxygen species in lethal reperfusion injury in vivo is still poorly understood because studies on efficacy of scavengers of reactive oxygen species against reperfusion injury have been highly equivocal.[29,30]

In the present in vivo study, the tyrosine kinase inhibitor genistein dose-dependently attenuated the cardioprotection by BMOV even when BMOV was administered before ischemia and genistein was administered after 45 min of ischemia (i.e. just before reperfusion). These findings are consistent with the concept that BMOV is dependent on an intact tyrosine kinase activity during reperfusion and also suggest that the tyrosine phosphorylation status is an important determinant of ischemia reperfusion damage. An increased phosphorylation of tyrosine residues has been proposed to afford protection against ischemia reperfusion damage via a number of subcellular actions. First, vanadate exerts insulinlike effects including enhanced stimulation of glucose transport and oxidation in the isolated rat heart, which might be due to its tyrosine phosphatase inhibitory actions.[23] Recent clinical trials indicate that a combination of glucose and insulin might increase the salvage of cardiomyocytes during early reperfusion.[31] The mechanism by which enhanced glucose utilization produces protection might be related to increased ATP production at the site of the sarcolemma (and perhaps the mitochondria) during the first few minutes of reperfusion (at a time when mitochondria have not yet resumed ATP production), thereby maintaining ion homeostasis and chaperoning the vulnerable cardiomyocytes into a phase in which the mitochondria resume ATP generation.[32] Another mechanism by which an increase in tyrosine phosphorylation may exert cardioprotection could involve opening of K^+_{ATP} channels, which is suggested by studies showing that ischemic preconditioning involves activation of tyrosine kinase and protein kinase C[2,3] and opening of K^+_{ATP} channels.[18] Although the sequence of involvement is still controversial,[33] studies in the rat heart suggest that kinases are principally involved early in preconditioning and act upstream of the K^+_{ATP} channels.[3,34,35] In accordance with this concept, we observed that the K^+_{ATP} channel blocker glibenclamide, which had no effect on infarct size per se, abolished the cardioprotection by BMOV, suggesting that opening of K^+_{ATP} channels is involved in the actions of BMOV. However, one could argue that glibenclamide antagonized the effects of BMOV by increasing myocardial susceptibility to ischemia (i.e., in a BMOV-independent manner), but that this went undetected in the control

infarct group because infarct size reaches a plateau at 60 min of coronary artery occlusion.[16] This scenario is, however, highly unlikely in view of a preliminary study from our laboratory in which we observed that the reduction in infarct size by ischemic preconditioning with three cycles of 3 min of coronary artery occlusion and interspersed by 5 min of reperfusion from 70 ± 1% (in sham rats) to 25 ± 4% ($P < 0.05$) was not affected by glibenclamide (infarct size 28 ± 8%).[36] Taken together, the findings in the present study are consistent with the concept that K^+_{ATP} channel activation during early reperfusion contributes to the protection by BMOV. Since glibenclamide blocks both the sarcolemmal and mitochondrial K^+_{ATP} channels,[37,38] future studies are needed to determine the involvement of mitochondrial versus sarcolemmal K^+_{ATP} channels in the protection against reperfusion injury by BMOV.

We have previously shown that a brief episode of intestinal ischemia can elicit remote preconditioning of the heart via a neurogenic pathway that induces protection via myocardial adenosine release and consequent receptor stimulation in the rat heart.[12,13] Because BMOV, when administered intravenously after a total coronary artery occlusion, cannot easily reach the jeopardized myocardium before reperfusion has been reinstated (rats lack a significant coronary collateral circulation), we hypothesized that activation of a neurogenic pathway could have contributed to the protective actions of BMOV. However, in the present study, we observed that the ganglion blocker hexamethonium had no effect on the cardioprotection by BMOV, suggesting that a neurogenic pathway is not involved in the cardioprotection by BMOV.

Clinical Relevance. In patients with an impending myocardial infarction, early restoration of blood flow to jeopardized ischemic myocardium is compulsory for limiting infarct size. Despite its necessity, several investigators[39,40,41] have suggested that reperfusion causes irreversible myocardial damage by itself, beyond that inflicted by ischemia alone. This "lethal reperfusion injury" implies the death of cardiomyocytes (which are still viable at the onset of reperfusion) as a direct result of sequelae initiated by reperfusion itself, thereby resulting in extension of myocardial infarction. Since in patients that encounter a myocardial infarction as the first symptom of ischemic heart disease pharmacotherapy can only be applied after the coronary artery has become occluded, there is a need for agents that are protective even when given after the onset of ischemia or just before reperfusion. Most cardioprotective agents developed to date require administration before the onset of ischemia to be effective.[30] The present study shows that BMOV, administered in a sufficiently high dose, is still highly cardioprotective when administered just before reperfusion.

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3.2 THE INFLUENCE OF PRESERVATION SOLUTION ON THE VIABILITY OF GRAFTS IN HAIR TRANSPLANTATION SURGERY

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SUMMARY

Hair transplantation techniques have changed in the last decades. Partial longitudinal follicular unit transplantation is a new hair transplantation technique, which differs from all other hair transplantation techniques by the size of the graft and therefore much more vulnerable grafts compared to the conventional hair transplantation grafts. In this study, we reveal the influence of the preservation solution on the viability of the grafts. We have extracted 15 hair transplantation grafts of 0.6 mm and 15 hair transplantation grafts of 0.7 mm from 3 different patients and investigated the influence of 2 commercially available preservation media, saline solution (Braun, Melsungen, Germany) and Ringer's lactate (Braun), on the viability of grafts and compared these solutions with the preservation solution developed by Hair Science Institute with trypan blue. The grafts stored in the preservation solution developed by Hair Science Institute showed a significant better viability compared with the 2 commercially available preservation media saline solution and Ringer's lactate. This study shows that a preservation solution could influence the viability of the grafts which could be essential for hair transplantations with small grafts such as in partial longitudinal follicular unit transplantation.

INTRODUCTION

Earlier studies revealed that in conventional hair transplantation techniques, such as the strip method, the preservation solution could influence the survival rate after implantation[1] and could reduce apoptosis in the grafts.[2] Crisóstomo[3] described the presence of oxidative stress in hair transplants. These studies showed that hair transplantation grafts are vulnerable, but do not reveal that in conventional hair transplantation procedures, the preservation solution is essential, since grafts, usually stored for a minimum period of 2 hours in saline solution before implantation, will regenerate new hairs.[4] However, hair transplantation techniques have changed dramatically in the last decades from full punch grafts via the strip method to follicle unit transplantations, which automatically means that the grafts become smaller and smaller.

Partial longitudinal follicular unit transplantation (PL-FUT) is a new hair transplantation technique, which enables us to transplant hairs with the preservation of the donor site. PL-FUT differs from all other hair transplantation techniques by the size of the graft.[5] The grafts used in PL-FUT are approximately 0.5–0.6 mm in diameter (surface area of 0.95 mm^2) and are approximately 2.5 times smaller than the micrografts of 0.8–1.0 mm in diameter (surface area of 2.54 mm^2) used in conventional hair transplantations. Consequently, the PL-FUT grafts are much more vulnerable compared to the conventional hair transplantation grafts.

This problem was already recognized in 2006 by Er et al,[6] who recommended not to implant sectioned hair follicle parts. He stated that the survival rate of the transected hair follicles is directly related to the level of transection and that the growth rate of the sectioned parts is not satisfactory and he stated that the hairs are thinner than the original follicles.[6]

Although Er et al[6] did not recommend to implant partial hair follicles because of the changed characteristics, we try to reveal the influence of the preservation solution on the viability of the grafts and, therefore, the characteristics of the regrown hairs.

In PL-FUT, we transplant a considerably smaller amount of tissue compared to conventional hair transplantation procedures; in our opinion, the preservation solution will influence the survival rate of the grafts and therefore hair growth. This hypothesis is based on:

1. The small size of the graft (0.5–0.6 mm).
2. Long-time interval between extraction and implantation (2–6 h).

MATERIALS AND METHODS

In this study, we have extracted grafts from 3 different hair transplantation patients. Per patient, 15 hair transplantation grafts of 0.6 mm and 15 hair transplantation grafts of 0.7 mm were extracted (Table 1).

We investigated the influence of 2 commercially available preservation media, saline solution (Braun, Melsungen, Germany) and Ringer's Lactate (Braun), on the viability of grafts and compared these solutions with the preservation solution developed by Hair Science Institute (HSI). This preservation solution contains sodium chloride, potassium chloride, magnesium sulfate, sodium phosphate, calcium chloride, glucose, sodium bicarbonate, sodium lactate, sodium pyruvate, human serum albumin, insulin, and the following ingredients: bis(maltolato) oxovanadium (BMOV) and monohydroxy ethylrutoside (HSI, Maastricht, The Netherlands).

Trypan blue can be used to determine nonviable tissue.[7] To compare the viability of the grafts, we stored the 0.6 mm and 0.7 mm micrografts in saline solution, Ringer's lactate, or our preservation solution for 4 hours. Afterward, we have left the grafts in a 0.1% trypan blue solution for 15 minutes (Fig. 1).

We have evaluated the viability of the grafts semi-quantitatively in the following levels (Table 1):

Dark blue	Low viability	**–**
Blue	Moderate viability	**–/+**
Light blue	High viability	**+**

	0.6-mm Grafts			0.7-mm Grafts		
Patient	Saline Solution (NaCl 0.9%)	Ringer's Lactate	HSI Solution	Saline Solution (NaCl 0.9%)	Ringer's Lactate	HSI Solution
1	**–**	**–/+**	**–/+**	**–**	**–/+**	**–/+**
2	**–**	**–/+**	**–/+**	**–**	**–/+**	**–/+**
3	**–**	**–/+**	**–/+**	**–**	**–/+**	**–/+**

Table 1: The Viability of Grafts in Hair Transplantation Surgery

RESULTS

In Figures 1 and 2, we can clearly see that the viability of the grafts is higher in Ringer's lactate compared to the saline solution, but the viability of the grafts is the highest in the preservation solution developed by HSI.

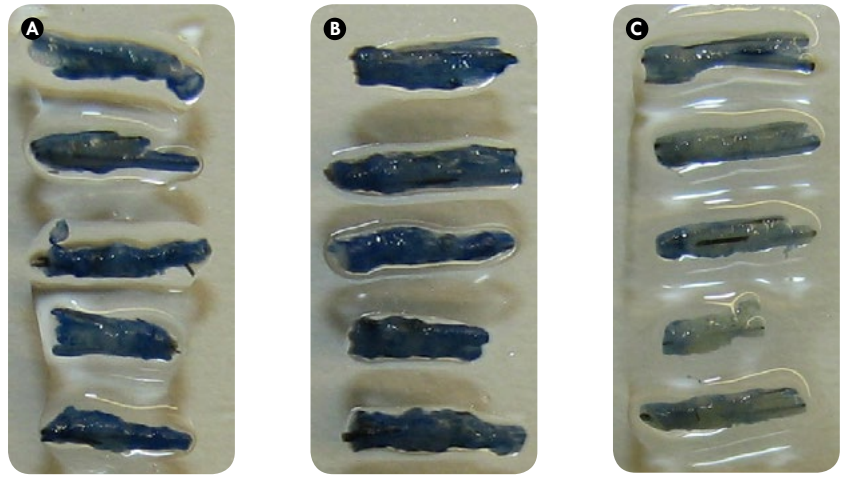

Figure 1: Grafts (0.7 mm) after 4 h in **(A)** *saline solution,* **(B)** *Ringer's lactate, and* **(C)** *preservation solution developed by HSI and 0.1% trypan blue solution for 15 min. The viability of the grafts is higher in Ringer's lactate compared to the saline solution, but the viability of the grafts is the highest in the preservation solution developed by HSI.*

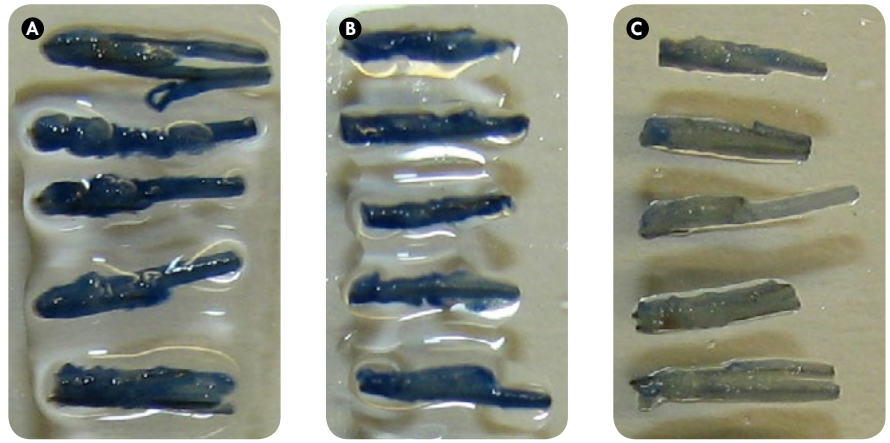

Figure 2: Grafts (0.6 mm) after 4 h in **(A)** *saline solution,* **(B)** *Ringer's lactate, and* **(C)** *preservation solution developed by HSI and 0.1% trypan blue solution for 15 min. The viability of the grafts is higher in Ringer's lactate compared to the saline solution, but the viability of the grafts is the highest in the preservation solution developed by HSI.*

DISCUSSION

Since Krugluger et al[2] has described the presence of apoptosis and Crisóstomo[3] revealed the relevance of oxidative stress in hair transplant grafts, the ingredients BMOV and monohydroxy ethylrutoside could have beneficial effects on the viability of the grafts.

Lebeau et al[8] and Ortolani et al[9] revealed that different flavonoids, such as rutosides, have beneficial effects on ischemia and reperfusion injury. BMOV has many interesting physiological actions but probably exerts its main influence in the prevention of apoptosis by its ability to block the tyrosine phosphorylation pathway.[10]

We revealed that a considerably amount of tissue will become not viable (Fig. 1) in saline solution. However, when using large grafts, the amount of viable tissue seems to be sufficient to regenerate new hair growth in conventional hair transplantation techniques,[4] This study clearly shows that a preservation solution is essential for hair transplantations with small grafts such as in PL-FUT. HSI's preservation solution differs significantly from saline solution, Ringer's lactate, and even from commercially available media used for keratinocyte culture. The addition of BMOV and rutosides will reduce oxidative stress, which is known to damage hair transplantation grafts.[3] Any other addition such as growth factors seems to be unnecessary as viability tests have shown excellent survival of the grafts.

In addition, we studied the survival rate of 0.6-mm grafts in patients. This study showed that all implanted grafts developed to full differentiated hairs with a normal diameter.[5] In contrast to the experiments of Er et al[6] our grafts differ in the way that the part of the follicle is taken in a longitudinal way.[5] As we have proven that follicular stem cells are located at different areas in the hair follicle, we believe that these hair transplantation grafts, which contain also stem cells, are responsible for complete regrowth of the hairs.

CONCLUSIONS

The use of a suitable preservation solution could be one of the crucial conditions in the success of all hair transplantation procedures with small grafts, such as PL-FUT.

Although we have not investigated the efficacy of the different individual ingredients and different combination of the ingredients, the described preservation solution works perfect for PL-FUT.

In theory, it can be possible that the same efficacy can be obtained with excluding one or more ingredients of the solution. It was not our aim to investigate the minimum composition, but only to reveal the influence of the preservation solution on the viability of the grafts. Nevertheless, in our opinion, we advise to use a suitable preservation solution for hair transplantation procedures with small grafts (under 0.8 mm).

Additional studies with (improved) preservation solutions need to be performed to evaluate the minimal quantity of tissue necessary to regenerate hair growth.

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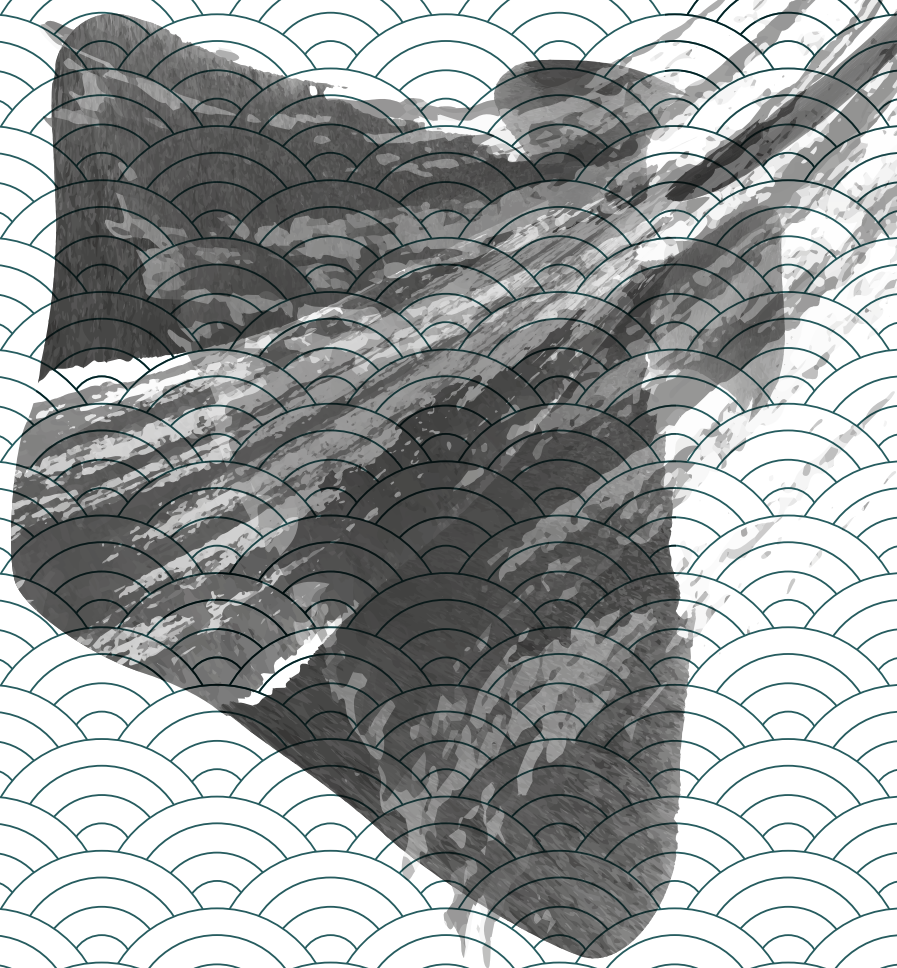


4

THE HAIR GROWTH POTENTIAL

OF FOLLICULAR STEM CELLS, PARTIAL FOLLICULAR UNITS AND THEIR CLINICAL APPLICATION IN HAIR RESTORATION

4.1 DONOR HAIR FOLLICLE PRESERVATION
BY PARTIAL FOLLICULAR UNIT EXTRACTION. A METHOD TO OPTIMIZE HAIR TRANSPLANTATION

Coen G. Gho, H.A. Martino Neumann

Gho, C. G. and H. A. Martino Neumann (2010). "Donor hair follicle preservation by partial follicular unit extraction. A method to optimize hair transplantation." J Dermatolog Treat 21(6): 337-349

ABSTRACT

Background

There are different stem cell pools located in the hair follicle. Objective: To try to determine whether follicular units can survive a partial extraction and whether this partial extracted follicular unit can regenerate new hairs. Methods: From five individuals, between 100 and 150 grafts were harvested from the occipital area of the scalp. Suitable grafts were implanted into the recipient area. Hair growth and characteristics in the donor area and the recipient area were observed at different intervals. Results: After 3 months, between 92.1% and 104.1% (mean 97.7%) of the partial follicular units in the donor sites survived and produced hairs with the same characteristics. After 1 year, 91.1–101.7% (mean 95.9%) of the implanted partial follicular units regenerated hair growth with the same characteristics as the hairs in the donor area. Conclusions: We revealed that extracted partial longitudinal follicular units transplanted to the recipient area can be used as complete follicular units to regenerate completely differentiated hair growth with the same characteristics as in the donor area. We also revealed that the partial follicular units in the donor area can survive and produce the same number of hairs with the same characteristics. This technique enables us to generate two hair follicles from one follicle with consistent results and preserve the donor area.

INTRODUCTION

Over 60% of men and 50% of women suffer from androgenetic alopecia.[1,2] Since this type of hair loss is a semi-natural process, and medication can only inhibit this temporarily, hair transplantation is the only method to restore hair permanently.

There are different techniques of hair transplantation, all with their advantages and disadvantages. The most common and known hair transplantation method is the so-called 'strip' method.[3] A strip of skin containing hair follicles is removed, cut into grafts and implanted in the recipient area. In recent years, new methods have developed, of which the most promising is the follicular unit extraction (FUE) method.[4] With this method, whole follicle units are extracted one by one and implanted one by one back into the recipient area. The FUE method is a major step towards perfecting hair transplantation.

Although the FUE method is more patient friendly and leaves only tiny scars compared to the strip method, which leaves visible linear scars at the donor area, the major disadvantage of both methods is that the extracted hair follicles are removed and the source of potential grafts will be consumed in time. Hair transplantations with the described methods will always be limited by the availability of donor hair follicles because no re-grow will occur in the donor area.The cosmetic result depends not only on the graft type (single-hair grafts or follicular units), the survival rate of the transplantation and the skill of the surgeon, but also on the number of grafts one can transplant.

To date, no multiplication of human hair follicles in vitro is possible. The only theoretical way to preserve a significant part of the donor hair follicles could partial FUE. This idea is not unrealistic and is supported by different experiments.[5,6] Kim and Choi[5] found that, in humans, the proximal part of the hair follicle cannot regenerate into a differentiated hair follicle, but the distal part of the follicle can, eventually resulting in a fully developed hair follicle.[7] Reynolds et al. found that, although the dermal papillae of humans cannot induce new hair growth, the sheath of the lower part of the hair follicle can.[6] These apparently contradictory results indicate that both proximal and distal areas of the hair follicle should contain follicular stem cells that can induce hair growth.

In an earlier study we revealed that hair follicles from skin biopsies expressed CK19 and Bcl-2 in cells from the upper to the lower third of the follicle.[8] Commo et al. also observed that distinct areas in the hair follicle from skin biopsies are positive for CK19.[9] The fact that these areas are also Bcl-2 positive and Bax negative is a strong indication for different follicular stem cell sites which can induce hair growth. Positivity for CK19 and Bcl-2 corresponds to infrequent cell division in these areas, as concluded from the absence of Ki-67 staining.[10,11] The fact that these cells are positive for Bcl-2 and CK 19, but Ki-67 and Bax negative, is a strong indication that they represent follicular stem cells in the hair follicle.

In case of partial longitudinal FUE, where follicular stem cells remain at the donor site as well as in the partial extracted follicle, a donor site capable of multiple hair transplantations should become possible.

The main objectives of this study are: 1) to determine the percentage of re-growth and characteristics of hairs from transplanted partial longitudinal follicular units in the recipient area; and 2) to determine the survival rate and the percentage of re-growth of the partial follicular units remaining in the donor area; the characteristics of the re-grown hairs are also evaluated.

			Number of grafts					
№	**Extracted**	**Complete follicular units**	**Partial longitudinal follicular units – suitable**		**Incomplete follicular units – unsuitable**		**Total number of visible hairs of the suitable grafts**	**Total number of visible hairs of the unsuitable grafts**
a	b	c	d		e		f	g
1	125	0	110	(88%)	15	(12%)	238	20
2	150	0	124	(83%)	26	(17%)	267	40
3	150	0	104	(69%)	46	(31%)	197	70
4	125	0	105	(84%)	20	(16%)	230	25
5	100	0	94	(94%)	6	(6%)	203	13
Mean	130	0	107.4	(83.6%)	22.6	(16.4%)	227	33.6

Table 1: Selection of the grafts.

		Number of visible hairs in outlined donor area			Re-growth in the donor area		
№	**Duration of the extraction in minutes (graft/min)**	**Before extraction**	**Directly after extraction**	**12 months after extraction**	**Total number of visible hairs of the suitable grafts**	**Total number of re-grown hairs**	**Percentage re-growth in the donor area (%)**
a	b	c	d	e	f	g	h
1	75 (1.7)	370	112	354	238	222	93.3
2	95 (1.6)	392	85	401	267	276	103.4
3	80 (1.9)	344	77	352	197	205	104.1
4	65 (1.9)	319	64	308	230	219	95.2
5	75 (1.3)	318	102	302	203	187	92.1
Mean	(1.68)	348.6	88	343.4	227	221.8	97.7

Table 2: Re-growth of the hairs in the donor area.

MATERIALS AND METHODS

Patients

Five healthy male individuals (aged 36–61 years, mean 44.8 years) (Table 1) who consulted the Hair Science Institute with proven androgenetic alopecia and gave their informed consent, participated in the study. The protocol was approved by the Institutional Review Board and the study was conducted according to the declaration of the Helsinki principles.

The technique

A. Preparation and outlining of the donor site. On the occipital side of the scalp, an area of 15 x 5 cm was shaved, disinfected with chlorhexidine 2% lotion and anaesthetized with lidocaine 2% with adrenaline (AstraZeneca). Within that area, 1.5 1.5 cm was outlined with an acupuncture needle dipped in semipermanent black pigment.

B. Counting of the hairs. The outlined area was photographed with a digital camera (Nikon E4800), and the hairs in the area were counted (Table 2, column c).

Figure 1: Close-up of the triple-waved tipped extraction needle.

C. Extraction of the partial longitudinal follicular units (grafts). At least 100 grafts were harvested with hollow triple-waved-tipped, partially blunt needles with an inner diameter of 0.6 mm (Figure 1) (Hair Science Institute®, Amsterdam, The Netherlands) (Table 1, column b). To extract a partial longitudinal follicular unit, we used the hair shafts as guidance for the needle. This enables us to extract a partially longitudinal follicular unit, even when the follicular unit is not in a perfect triangular configuration.

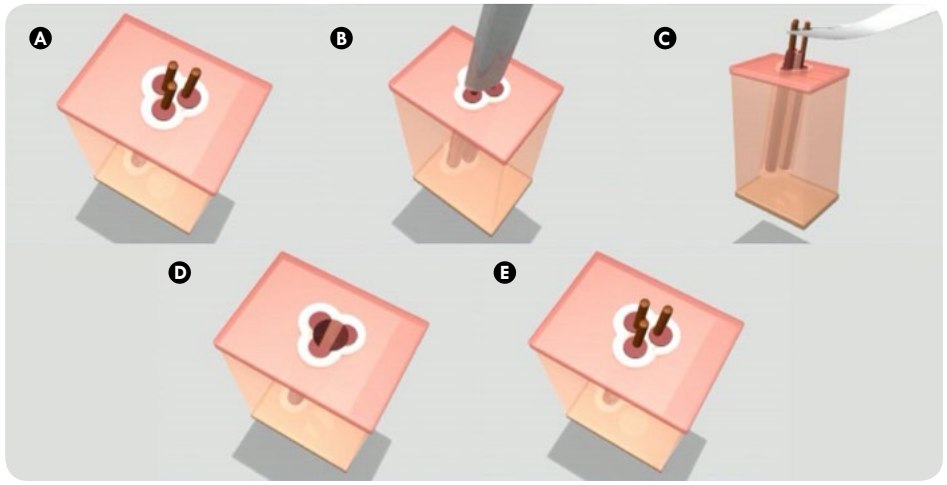


Figure 2: Illustrations of the procedure in the donor area. **(A)** *The whole follicular unit.* **(B)** *Extraction of the longitudinal partial follicular unit with a 0.6 mm needle.* **(C)** *Extraction of the longitudinal partial follicular unit with micro-surgical forceps.* **(D)** *Part of the follicular unit which is left behind.* **(E)** *Re-growth in the donor area*

Figure 2A shows a follicular unit containing the visible hairs (brown), hair follicle (dark pink) and connective tissue (white). The needle is placed around the visible hairs and rotated until the grafts are detached from the dermis (Figures 2A and B). The grafts are extracted with micro-surgical forceps (Figure 2C). The aim of the extraction is to remove only a part of the follicle unit, containing follicle and connective tissue from several hair follicles, and leave sufficient follicle unit tissue behind to regenerate hairs (Figures 2D and E). After the extraction, Fucidin Cream® (Leo Pharma, Breda, The Netherlands) was applied on the donor area.

D. Selection of the grafts. After the extraction, the graftswere visually evaluated, selected and divided in three groups: complete follicular units (Figure 3A) (Table I, column c); partial longitudinal follicular units (suitable grafts) (Figure 3C) (Table 1, column d); and incomplete follicular units (unsuitable grafts), which did not contain sufficient tissue (Figure 3E) (Table 1, column e). By using a magnifier (2x) we were able to distinguish between partial longitudinal follicular units, which show terminal hairs at the border and do not contain surrounding tissue, and complete follicular units, which contain complete hair follicles with surrounding tissue. We illustrate the difference between a complete follicular unit (Figure 3A) (Table 1, column c) and a partial longitudinal follicular unit (suitable grafts) (Figure 3C) (Table 1, column d) with Figure 3B, which shows a haematoxylin & eosin (HE)-stained transversal sectioned complete follicular unit (10x), and Figure 3D, which shows a HE-stained transversal sectioned partial longitudinal follicular unit (20x), where a considerable part of the original follicular unit is left behind at the donor site.

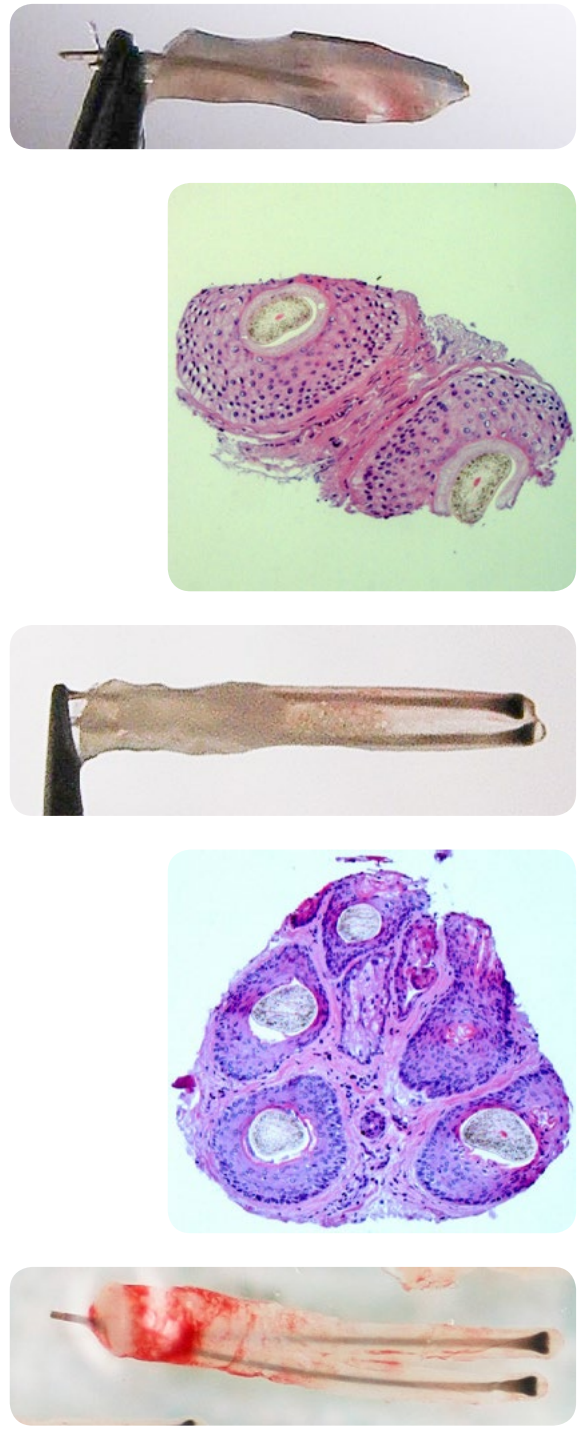


Figure 3: The grafts **(A)** *Complete follicular unit grafts.* **(B)** *Haematoxylin & Eosin (HE) stained transversal sectioned complete follicular unit (10x).* **(C)** *Partial longitudinal follicular unit grafts.* **(D)** *Haematoxylin & Eosin (HE) stained transversal sectioned partial longitudinal follicular unit (20x), where a considerable part of the follicular unit is left behind at the donor site.* **(E)** *Grafts which contain not sufficient tissue.*

Figure 4 illustrates the difference between a complete follicular unit (green circle) and a partial longitudinal follicular unit (blue circle) in a transversal section of the skin.

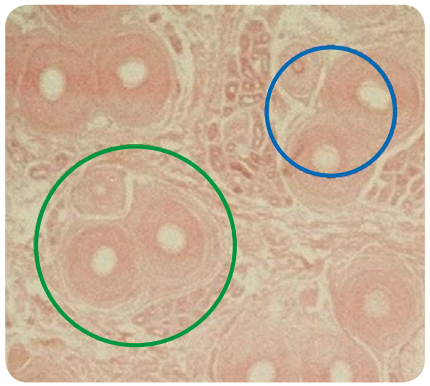


Figure 4: The difference between a complete follicular unit (green circle) and a partial longitudinal follicular unit (blue circle) in a transversal section of the skin.

Suitable longitudinal partial follicular units (Figure 3C) were visually selected (Table 1, column d) and stored in the preservative medium for 2 hours until implantation. The medium is composed of the following ingredients: sodium chloride, potassium chloride, magnesium sulphate, sodium phosphate, calcium chloride, glucose, sodium bicarbonate, sodium lactate, sodium pyruvate, human serum albumin, insulin, bis(maltolato)oxovanadium (BMOV) and α-tocopherol (vitamin E) (Hair Science Institute).

E. Counting the number of hairs within the suitable and unsuitable grafts. The total number of visible hairs in the selected partial longitudinal follicular units (suitable grafts) (Table 1, column f), as well as the total number of visible hairs in the unsuitable incomplete follicular units, was counted (Table 1, column g).

F. Measurement of the hair diameter from hairs derived from the donor area. From the suitable grafts, 10 hairs were measured with an electronic digital micrometer to determine their diameter (Table 3, column b).

G. Measurement of the hair diameter from hairs in the donor area. After 12 months, 10 hairs in the outlined donor area were measured with an electronic digital micrometer to determine their diameter (Table 3, column c).

H. Preparation and outlining the recipient area. The recipient area was disinfected with chlorhexidine 2% lotion and anaesthetized with lidocaine 2% with adrenaline (AstraZeneca). Within this area, 2.5 x 2.5cm was outlined with an acupuncture needle dipped in semipermanent black pigment. Miniscule holes were made with a hollow needle with an inner diameter of 0.6mm (Hair Science Institute). Remaining bald tissue was removed with micro-surgical forceps (Figure 5A).

Average diameter of 10 hairs

No	Before extraction (µm)	12 months after the extraction (µm)
a	b	c
1	55	54
2	94	93
3	56	55
4	82	80
5	65	63
Mean	70.4	69

Table 3: Characteristics of the hairs in the donor area.

I. Implantation of the grafts. After preparation of the recipient area, the selected grafts were implanted with micro-surgical forceps (Figure 5B) (Table IV, column b). The aim of the implantation is to implant sufficient follicle and connective tissue from several hair follicles to regenerate hair growth (Figure 5C).

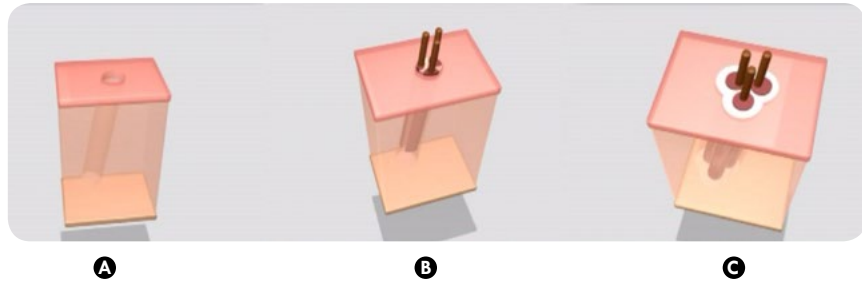


Figure 5: Illustrations of the procedure in the recipient area. **(A)** *Holes in the recipient area, made with a same sized needle.* **(B)** *Implantation of the longitudinal partial follicular unit with micro-surgical forceps.* **(C)** *Re-growth in the recipient area.*

		Number of visible hairs in outlined recipient area				Re-growth in the recipient area		
№	**Partial longitudinal follicular units – implanted**	**Implantation time in minutes (graft/min)**		**Before implantation**	**12 months after implantation**	**Total number of visible hairs of the suitable grafts**	**Total number of re-grown hairs**	**Percentage re-growth in the donor area**
a	b	c		d	e	f	g	h
1	110	21	(5.2)	37	279	238	242	101.7
2	124	25	(5.0)	52	314	267	262	98.1
3	104	17	(6.1)	70	263	197	193	98.0
4	105	21	(5.0)	47	253	230	206	89.6
5	94	15	(6.2)	62	247	203	185	91.1
Mean	107.4	19.8	(5.5)	53.6	271.2	227	217.6	95.9

Table 4: Re-growth of the hairs in the recipient area.

	Average diameter of 10 hairs	
No	**Before extraction (µm)**	**12 months after the extraction (µm)**
a	b	c
1	55	53
2	94	91
3	56	55
4	82	80
5	65	66
Mean	70.4	69

Table 5: Characteristics of the hairs in the recipient area.

Evaluation, calculation and follow-up

A. Evaluation and calculation of re-growth in the donor area. The outlined area at the donor site was photographed before the extraction (Figure 6A) and the visible hairs in the outlined area were counted before and directly after the extraction (Figure 6B) (Table 2, columns c and d). At intervals of 1 week (Figure 6C), 3 and 12 months after the extraction, the outlined area was shaved and photographed. After 12 months, the visible hairs in the outlined area were also counted (Table 2, column e)

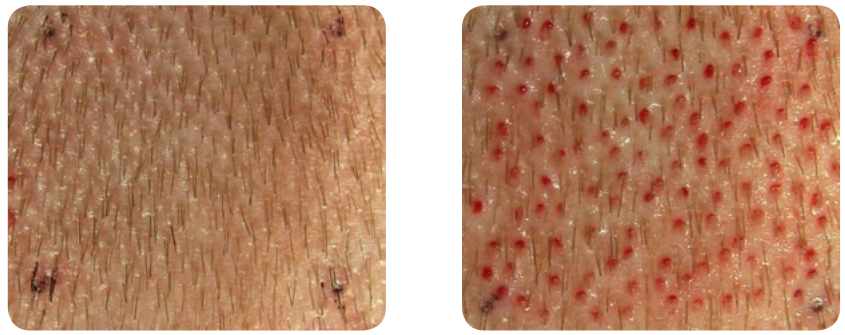


Figure 6: Evaluation of the donor area. **(A)** *The outlined area at the donor site before the extraction.* **(B)** *The outlined area at the donor site directly after the extraction.* **(C)** *The outlined area at the donor site 9 days after the extraction.* **(D)** *The outlined area at the donor site 1 month after the extraction.*

The number of re-grown hairs in the donor area (Table 2, column g) was calculated as follows: the total number of visible hairs after 12 months in the outlined area (Table 2, column e) minus the number of hairs which were left in the donor area (Table 2, column d) minus the total number of visible hairs in the unsuitable incomplete follicular units (Table 1, column g).

The survival rate in the donor area (Table 2, column h) was calculated as follows: since the total number of visible hairs in the suitable grafts (Table 2,column f) are supposed to be the hairs which are 'extracted' and suitable to regenerate new hairs and the total number of re-grown hairs in the donor area (Table 2, column g) are supposed to be the hairs which have been preserved after extraction, the survival rate in the donor area (Table 2, column h) was calculated as follows: the total number of re-grown hairs in the donor area (Table 2, column g) divided by the total number of visible hairs in the suitable grafts (Table 2, column f).

B. Evaluation of the characteristics in the donor area. From the suitable grafts, the diameter of 10 hairs was measured with an electronic digital micrometer and the average calculated (Table 3, column b). Twelve months after the extraction, the diameter of 10 hairs in the same area was measured again by an electronic digital micrometer and the average calculated (Table 3, column c).

C. Evaluation and calculation of re-growth in the recipient area. The outlined recipient area was photographed before and at intervals of 1 week, and 3 and 12 months after implantation. The visible hairs in the outlined area were counted before (Table 4, column d) and 12 months after the implantation (Table 4, column e).

The number of re-grown hairs in the outlined recipient area (Table 4, column g) was calculated as follows: the total number of visible hairs after 12 months in the outlined area (Table 4 column e) minus the number of hairs which was already present in the recipient area (Table 4, column d). The survival rate in the outlined recipient area (Table 4, column h) was calculated as follows: the total number of re-grown hairs in the outlined recipient area (Table 4, column g) divided by the total number of visible hairs in the suitable grafts (Table 4, column f).

D. Evaluation of the characteristics in the recipient area. The diameter of 10 hairs in the recipient area was measured by a micrometer 12 months after implantation and the average calculated (Table 5, column c). This was compared with the average diameter of 10 hairs from the extracted partial follicular units (Table 5, column b).

E. Multiplication of hairs. The number of extra hairs, and therefore the multiplied hairs (Table 6, column f) was calculated as follows: the number of re-grown hairs in the donor area (Table 6, column c) plus the number of re-grown hairs in the outlined recipient area (Table 6, column d) minus the total number of visible hairs in the suitable grafts (Table 6, column e).

The multiplication rate (Table 6, column g) can be calculated in different ways: the number of extra (multiplied) hairs (Table 6, column f) divided by the total number of visible hairs in the suitable grafts (Table 1, column e) or the percentage of re-grown hairs in the donor area (Table 2, column h) plus the percentage of re-grown hairs in the outlined recipient area (Table 4, column h) minus 100%.

RESULTS

Grafts

In this study of five patients, between 100 and 150 grafts (mean 130 grafts) per patient were extracted (Table 1, column b). There were no grafts that contained complete follicular units (Table 1, column c). Between 69% and 94% (mean 83.6%) of the extracted grafts contained partial follicular units and therefore were suitable to be implanted in the recipient area (Table 1, column d). The suitable grafts contained between 197 and 267 visible hairs (mean 227 hairs) (Table 1, column f). Between 6% and 31% (mean 16.4%) of the extracted grafts were not used for implantation in the recipient area (Table 1, column e). Unsuitable grafts contained between 13 and 70 visible hairs (mean 33.6 hairs) (Table 1, column g).

Re-growth of the hairs in the donor area

The extraction time varied between 1.3 and 1.9 grafts (mean 1.68 grafts) per minute (Table 2, column b). The number of hairs in the outlined area before extraction varied between 318 and 392 hairs (mean 348.6) (Table 2, column c) and between 64 and 112 hairs (mean 88 hairs) were visible directly after extraction (Table 2, column d). Twelve months after extraction, between 302 and 401 hairs (mean 343.4 hairs) were visible in the outlined donor area (Table 2, column e).

If we assume that the number of hairs in the outlined area will remain the same, the percentage of regrown hairs varies between 92.1% and 104.1% (mean 97.7%) (Table 2, column h).

№	**Hairs left in the donor area**	**Number of re-grown hairs in the outlined donor area**	**Number of re-grown hairs in the outlined recipient area**	**Total number of visible hairs of the suitable grafts**	**Multiplication**	**Multiplication rate (%)**
a	b	c	d	e	f	g
1	132	222	242	238	226	95
2	125	276	262	267	271	101.5
3	147	205	193	197	201	102.1
4	89	219	206	230	195	84.8
5	115	187	185	203	169	83.2
Mean	121.6	221.8	217.6	227	212.4	93.3

Table 6: Table VI. Multiplication of the hairs.

The close-up picture of the donor site 1 week after extraction (Figure 7) shows re-growth in the donor area. The circles most probably show where the grafts were harvested since the area around the hairs is pinkish and these hairs are shorter compared with the surrounding hairs. The influence of extraction of unsuitable grafts (Table 1, column g) on the re-growth is minimal, since the number of unsuitable grafts is considerably smaller than the suitable grafts (Table 1, column f). In the blue circles small hairs are growing out. These circles show the re-growth of the hairs in the donor graft sites. The red circles do not contain visible hairs. However, in these donor-graft sites, small black points are visible. Evaluation after 3 and 12 months revealed that there were no bald spots visible anymore. This could mean that too much tissue had been taken to reveal re-growth within 1 week but that re-growth would be visible in successive weeks. Figures 6A–D, where the outlined area at the donor site has been evaluated before (Figure 6A), directly after (Figure 6B), 1 week after (Figure 6C) and 1 month after (Figure 6D) the extraction, confirmed this. The average hair diameter of the patients varied between 55 and 94 mm (mean 70.4 mm) (Table 3, column b). After 12 months, the average hair diameter of the patients varied between 54 and 93 mm (mean 69 mm) (Table 3, column c).

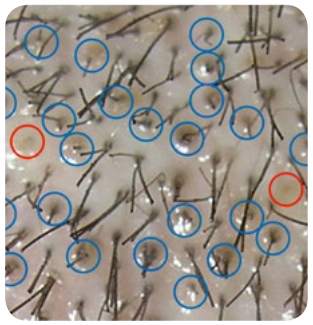


Figure 7: Close-up picture of the donor-site one week after extraction of the longitudinal parts of the follicular units. The pink spots shows where the grafts were taken. In these pink spots small hairs growing out (blue circles) are visible. This shows the re-growth of the hairs in the donor graft sites. Some pink spots do not contain small hairs (red circles). In these donor-grafts sites, probably too much tissue is taken to reveal re-growth after a week. However, there is a possibility that there will be re-growth after months.

Re-growth of the hairs in the recipient area

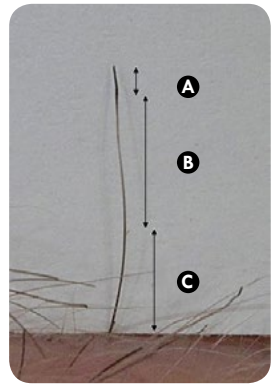
Between 94 and 124 (mean 107.4) suitable grafts were implanted in the outlined recipient area (Table IV, column b). The implantation time varied between 5.0 and 6.2 grafts (mean 5.5 grafts) per minute (Table IV, column c). The number of hairs in the outlined area before implantation varied between 37 and 70 hairs (mean 53.6) (Table IV, column d) and 12 months after implantation between 247 and 314 hairs (mean 271.2 hairs) were visible in the outlined recipient area (Table IV, column e). Assuming that the number of hairs in the outlined area remains the same, re-growth in the outlined recipient area would be between 89.6% and 101.7% hairs (mean 95.9%) (Table IV, column h).

Characteristics of the hairs in the recipient area

The average hair diameter of the patients varied between 55 and 94 mm (mean 70.4 mm) (Table V, column b). After 12 months, the average hair diameter of the patients varied between 53 and 91 mm (mean 69 mm) (Table V, column c).

Most small hairs from the implanted grafts fall out during the first weeks after implantation, although some hairs are able to continue growing. Figure 8 does not show re-growth in the recipient area in general, but demonstrates the dynamic re-growth process of a partial follicular unit in the recipient area during the first 3 months after implantation. The partial follicular unit is able to produce a differentiated hair, initially. The re-grown hair is thinner than normal (Figure 8, part B), but after 3 months the hair has developed into hair with the same diameter and visible characteristics (Figure 8, part C) as in the donor area (Figure 8, part A).

Figure 8: A re-grown hair in the recipient area after 3 months. This picture shows that after implantation, the partial follicular unit is able to produce a differentiated hair, but in the first period after implantation, the re-grown hair is thinner than normal ***(B)****; after 3 months this hair has developed into a hair with the same characteristics, such as the diameter* ***(C)*** *in the donor area* ***(A)***.



Multiplication of the hairs

If we assume that the number of hairs left behind in the donor area (Table 6, column b) were the visible hairs directly after the extraction (Table 2, column d) plus the visible hairs in the unsuitable incomplete follicular unit grafts (Table 1, column g), the number of hairs which are multiplied varied between 169 and 271 hairs (mean 212.4 hairs) (Table 6, column f). This means a multiplication rate between 83.2% and 102.1% (mean 93.3%) (Table 6, column g).

Statistical analysis

Since only five patients participated in this study, no statistical analysis was performed.

DISCUSSION

The grafts

In our study, the diameters of the individual hair follicles of the patients were large. Therefore, the follicular units of these patients, containing at least two hairs, are larger than the diameter of the extraction needle (diameter of 0.6 mm) used for harvesting the grafts. As a result, in this study, there was not one graft extracted which contained a complete follicle unit.

Re-growth in the donor area

Figure 9 shows pictures of the donor area of a patient before (Figure 9A), directly after (Figure 9B) and 3 months after (Figure 9C) harvesting the grafts. After evaluation of the five patients in this study, almost all hair follicles in the donor site produced a hair after 12 months. In two cases, the number of hairs increased, probably due to invisible telogen hair follicles, which were not visible after extraction, but produced hairs in the successive period.

Since some hairs which were present after extracting the grafts were still present in the donor area when measuring the diameter after 12 months, these preexisting hairs could also be measured instead of the hairs which had re-grown. Therefore, the average diameter after 12 months could be influenced by these pre-existing hairs. However, since the average diameter of the hairs in the donor area after 12 months was not reduced compared to the hairs in the grafts which had been measured, we could assume that the influence of the existing hairs is minimal.

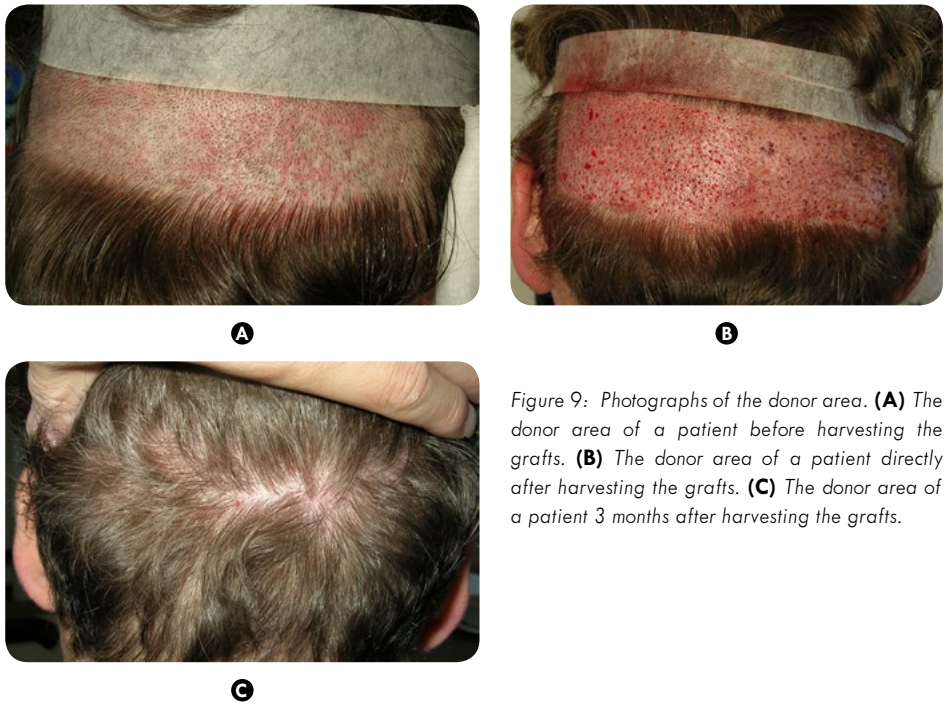


Figure 9: Photographs of the donor area. **(A)** *The donor area of a patient before harvesting the grafts.* **(B)** *The donor area of a patient directly after harvesting the grafts.* **(C)** *The donor area of a patient 3 months after harvesting the grafts.*

Re-growth in the recipient area

After evaluation of the five patients, it was observed after 12 months that almost all implanted grafts produced a hair in the recipient site. In one case, the number of hairs increased, probably due to invisible telogen hair follicles, which were not visible directly after the implantation, but produced a hair a few months after implantation.

Since some hairs were present the recipient area before implantation, these pre-existing hairs could also be measured instead of the hairs from the grafts which had implanted. Therefore, the average diameter after 12 months could be influenced by these preexisting hairs. However, since the average diameter of the hairs in the recipient area after 12 months was not reduced compared to the hairs in the grafts, which had been measured, and the number of pre-existing hairs was considerably lower compared to the implanted hairs, we could assume that the influence of the existing hairs is minimal.

Therefore, this study shows that:

1. Extracted partial longitudinal follicular units containing viable follicular stem cells with connective tissue transplanted to the recipient area can be used as complete follicular units to regenerate completely differentiated hair growth with the same diameter and characteristics as hair in the donor area.

2. The partial follicular units which remain in the dermis in the donor area can survive and produce the almost same number of hairs with the same diameter and characteristics when a longitudinal part of this follicular unit is extracted.

Our clinical data are in correspondence with the findings of Kim and Choi[5] and Reynolds et al[6]. We have proven that two hair follicles can be generated from one as long as only a part of the follicle is dissected from the original source. However, it is essential to realize that harvested partial follicular units can be obtained as:

- transversal, as published by Kim and Choi[5]
- longitudinal, as proposed in this study.

It is also important to realize that, practically, harvesting partial follicular units with the preservation of the donor follicular units can only be obtained in large quantities if they are from the longitudinal type.

In this study, we were not able to perform immunohistochemical experiments because our group of individuals underwent a standard hair transplant procedure. However, from our and Kim's experiments, we can calculate the multiplication rate from the regrowth of the harvest follicular stem cells at the recipient site and re-growth of the left follicular stem cells at the donor site as follows: the percentage of re-grown hairs in the donor area (Table 2, column h) plus the percentage of re-grown hairs in the outlined recipient area (Table 4, column h) minus 100%. These mathematical calculations are in full correlation with our clinical results.

Our clinical results concerning the re-growth of partial hair follicles are not in correlation with other clinical studies, such as the study of Er et al. Er et al. recommended not implanting sectioned hair follicle parts. They stated that the survival rate of the transected hair follicles is directly related to the level of transection and that the growth rate of the sectioned parts is not satisfactory and are thinner than the original follicles[12].

Earlier studies revealed that in conventional hair transplantation techniques, such as the strip-method, the preservative medium could influence the survival rate after implantation[13] and could reduce apoptosis in the grafts[14]. The smaller the amount of tissue transplanted the more influence the preservative medium had on the viability and apoptosis of the transplanted tissue, the survival rate of the grafts and therefore hair growth. Since the amount of tissue in our grafts is considerably smaller than with conventional hair transplantation methods, the influence of the preservative medium could be important. We used a medium which contained anti-apoptotic compounds such as BMOV, anti-oxidants such as vitamin E and growth-stimulating factors. This could be one of the factors why, in this study, we were able to use only longitudinal parts of follicular units with a maximum diameter of 0.6 mm instead of whole follicular units to regenerate new differentiated hairs in the recipient area with the same characteristics, such as the diameter, as hair in the donor area.

If too much follicle tissue has been removed from the donor area, only the graft will regenerate a new hair. If insufficient follicle unit tissue is removed from the donor area, only this follicle unit in the donor area will be capable of producing hairs. Therefore, the amount of tissue extracted from the donor follicular unit is vitally important for hair growth in both donor and recipient area. To minimize the variability of the amount of tissue extracted, instruments such as conventional punch needles were not suitable as in most cases too much tissue was extracted (non-published data).

Besides the variability of the amount of extracted tissue, conventional punch needles also damaged the grafts. Since the amount of tissue in our grafts was considerably smaller than the conventional hair transplantation methods, the damage to the grafts had considerably more influence on the survival rate of the grafts in our studies. Therefore, we had to develop new wave-tipped extraction needles of 0.5 and 0.6 mm (Figure 1). These needles use the hair shafts as a guide to extract longitudinal parts of the follicular unit which contain sufficient tissue to regenerate new differentiated hairs in the recipient area. The diameter of the grafts which contain the longitudinal parts of the follicular units extracted from the donor area varies between 0.5 mm and 0.6 mm. Since the diameter of a normal hair follicle is between 0.4 mm and 0.7 mm, and a follicular unit consists of at least two hair follicles, the needle is able to leave sufficient tissue behind to preserve the follicular unit in the donor area (Figure 4). Furthermore, this needle minimizes the damage to the grafts as well as the tissue in the donor area.

Differences with other transplantation techniques

The ideal hair transplantation should fulfil the following objectives:

1. An excellent cosmetic outcome.
2. 100% hair re-growth of the transplanted hair follicles.
3. 100% preservation and therefore an endless source of donor hair follicles.
4. No scarring.
5. A safe and comfortable procedure.
6. Short treatment duration.
7. No recovery time.
8. Not expensive.

Of all available transplantation techniques, there is no technique which fulfils all mentioned criteria. The main difference between our technique of partial longitudinal FUE compared with the other hair transplantation techniques is the preservation of the donor hair follicles without scarring.

In contrast to traditional hair transplantation techniques, which require a strip removal with a depth of 1–1.5 cm to obtain the hair follicles, we showed that successful transplantation is feasible using longitudinal partial follicular units with a diameter of 0.5–0.6 mm and 5–6 mm in length. Owing to minimal skin and tissue removal, there is minimal to no scarring, pain, or other post-surgical trauma such as nerve and vascular damage, nor is there the possibility that the linear scar will 'stretch out' over time. Absolutely no stitches or bandages are required.

FUE is another technique where the whole follicular unit is transplanted, without leaving sufficient tissue behind to regenerate a new hair or follicular unit. However, since the total follicular unit is extracted, this results in small scars in the donor area (Figure 10A).

With longitudinal partial follicular unit transplantation, parts of the follicular units remain in the dermis in the donor area. After longitudinal parts of these follicular units are extracted, they will survive and produce the same number of hairs with the same diameter and characteristics. These follicular units in the donor area can be used again in consecutive treatments (Figure 10B).

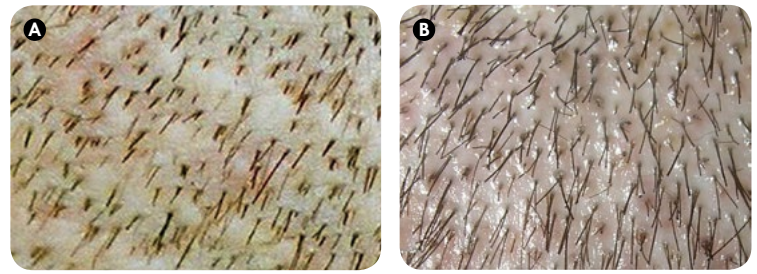


Figure 10: **(A)** *Small scars in the donor area due to follicular unit extraction (FUE).* **(B)** *Close-up of the donor site 1 week after extraction of the longitudinal parts of the follicular units. The parts of the follicular units that remain in the dermis in the donor area produce hair with the same characteristics, such as the diameter.*

Another difference is the progression of hair growth. In traditional hair transplantation, the majority of implanted hairs would fall out quickly after implantation and re-growth would occur within 3–6 months. In our study, re-growth started 5–8 months after implantation, but could sometimes take more than 12 months. This phenomenon was revealed in other hair transplantation studies with dissected hair follicles[15].

The weakness of this study is the limited number of patients. We also included patients who had had previous (traditional) hair transplantation. Therefore, a larger group of patients is necessary to study the real clinical relevance of this technique.

Longitudinal partial follicular unit transplantation is a very labor intensive procedure and to transplant sufficient grafts takes a full day. This technique may represent the first reliable patient-friendly method to generate two hair follicles from one hair follicle with consistent results and preserve the donor area. This technique is therefore suitable for people with a very limited donor area, the most extreme example of this being burn victims.

Acknowledgement
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4.2 IMPROVED HAIR RESTORATION METHOD FOR BURNS

Coen G.Gho, H.A. Martino Neumann

Gho, C. G. and H. A. M. Neumann (2011). "Improved hair restoration method for burns." Burns 37(3): 427-433.

ABSTRACT

Background
Extracted partial longitudinal follicular units can be used as complete follicular units to regenerate completely differentiated hair growth. The partial follicular units that remained in the dermis in the donor area can survive and produce hairs. This technique enables us to multiply hair follicles in vivo, while preserving the donor area and therefore is suitable in persons, who have a relative small donor area compared to the recipient area, as in scalp burns.

Objectives
With this study, we try to determine if partial longitudinal follicular unit transplantation (PL-FUT) can be used for facial and/or scalp burns.

Materials and methods
Four burn victims (age 22–39 years, mean 27.75 years) were treated in the face (eyebrows, and beard) and/or on the scalp with PL-FUT. The grafts were harvested with hollow wave-tipped needles with an inner diameter of 0.6 mm from the occipital area of the scalp. The suitable longitudinal partial follicular units were impregnated with a preservative medium, and implanted into the recipient area. Hair growth in the donor area as well as the recipient area was observed before treatment, and at intervals of 1 week, 3 months and 1 year after the treatment.

Results
After evaluation of the donor area, sometimes a few little white spots were visible, but almost all hair follicles in the donor site re-produce hairs after 2 years. All treated patients had satisfactory or very satisfactory cosmetic results in the treated area.

Conclusions
Partial longitudinal follicular unit transplantation (PL-FUT) may represent the firstreliable patient-friendlymethod to generate two hair follicles from one hair follicle with consistent results and preservation of the donor area. Therefore, this method is very suitable for people with facial and/or scalp burns.

INTRODUCTION

The head, especially the scalp and face, plays a central role in the psychological as well as physical self-esteem of the individual. It transmits expressions and emotions, communicates feelings and allows for individual identity. Facial burns disrupt these anatomical and functional structures creating pain, deformity, swelling and contractures that may lead to lasting physical and psychological problems.[1]

Although the extent of psychological trauma depends on the gender of the patient, age at the time of burn, stage of adolescence, intelligence and on the position and degree of burn, in general, facial- and scalp burns a significantly negative effect on the self-esteem of the patient.[2]

Therefore, reconstructive treatments for facial- as well as the scalp burns are very important for the physical as well as physiological well-being of the patient.

Almost one-third of all burns are facial burns.[3] Because hair on the face and scalp facial hair and scalp play a major role in cosmesis and expression of the individual, especially, hair restoration of the face (eyebrows, beard, etc.) and scalp may be beneficial for the self-esteem of the patient. Restoration of eyebrows, beard and scalp is possible with hair transplantation techniques. In addition to hair transplantation, scalp reductions are also commonly used in the treatment of facial and scalp burns.[4]

The ideal hair transplantation in burns should fulfil the following objectives:

1. excellent cosmetic outcome;
2. total hair re-growth of the transplanted hair follicles;
3. complete preservation and, therefore, endless source of donor hair follicles;
4. no scarring;
5. safe and comfortable procedure;
6. short treatment duration;
7. no recovery time; and
8. inexpensive.

Of all available hair transplantation techniques, there is no technique which fulfils all the mentioned criteria. There are different techniques of hair transplantation, with their advantages and disadvantages. The most common and known hair transplantation method is the so-called 'strip' method.[5] A strip of skin containing hair follicles is removed, cut into grafts and implanted in the recipient area. In the past years, new methods have developed of which the most promising is the follicle unit extraction (FUE) method.[6] With this method, whole follicle units are extracted one by one and implanted one by one back into the recipient area. Although the FUE method is more patient friendly and leaves only tiny scars compared with the strip

method, which leaves visible linear scars at the donor area, the major disadvantage of both methods is that the extracted hair follicles are removed and the source of potential grafts will be consumed in time. The cosmetic result depends not only on the grafts type (single hair grafts or follicular units), the survival rate of the transplantation and the skill of the surgeon, but also on the number of grafts one can transplant. Besides hair transplantation, scalp reduction is also commonly used in the treatment of facial and scalp burns.[4]

In burn victims, due to depletion of the donor area, especially when the donor area is also burned, hair transplantations with the described methods will always limited by the availability of donor hair follicles, because no re-growth will occur in the donor area, and therefore limits the possibilities of hair transplantations in burn victims.

The only way to preserve a significant part of the donor hair follicles could be partial FUE. This idea is not unrealistic and is supported by different experiments.[7,8] Kim and Choi[7] found that, in humans, the proximal part of the hair follicle cannot regenerate into a differentiated hair follicle, but the distal part of the follicle can, eventually resulting in a fully developed hair follicle.[9] Reynolds et al. found that, although the dermal papillae of humans cannot induce new hair growth, the sheath of the lower part of the hair follicle can.[8] These apparently contradictory results indicate that both the proximal and distal areas of the hair follicle should contain follicular stem cells that can induce hair growth.[10]

Partial follicular units can be obtained as: transversal[7] and longitudinal.[11] We report here about the possibility to use partial longitudinal follicular unit transplantation (PL-FUT) for burn patients. With this technique, follicular stem cells remain at the donor site as well in the extracted partial follicle. In this case, two hair follicles can be generated from one as long as only a part of the follicle is dissected from the original source.

It is also important to realise that, practically, harvesting partial follicular units with the preservation of the donor follicular units can only be obtained in large quantities if they are from the longitudinal type.

PL-FUT represents the first reliable patient-friendly method to generate two hair follicles from one hair follicle with consistent results and preserve the donor area. This technique could, therefore, be suitable for people with verylimited donor area, for example, burn victims. In this article, we reveal the possibilities of the PL-FUT for facial and scalp burns.

MATERIALS AND METHODS

1.1 Patients

Four burn victims (age between 22 and 39 years), who consulted Hair Science Institute with facial and scalp burns, have been treated in the face as well as on the scalp with PL-FUT (Table 1). In this article, we will discuss these patients.

Case	Initials	Date of birth (age)	Treatment	Treatment date	Location	Number of grafts
1	**KZ**	2-8-1986 (23)	1	29/30-06-2005	eyebrows, scalp	595 + 502
			2	08/09-03-2006	scalp	572 + 675
			3	04-04-2007	scalp	852
			4	15-04-2008	scalp	662
2	**MS**	29-4-1986 (24)	1	24-04-2006	eyebrows	300
3	**HW**	12-10-1969 (40)	1	15-08-2007	scalp	300
4	**BT**	22-7-1970 (29)	1	23-10-2008	beard	600

Table 1: Patients

1.2 PL-FUT

1.2.1.Preparation of the donor site

On the occipital side of the scalp, an area of 15 x 5 cm was shaved, disinfected with chloorhexidine 2% lotion and anaesthetised with lidocaine 2% with adrenaline (AstraZeneca).

1.2.2. Extraction of the partial longitudinal follicular units (grafts)

The grafts were harvested with hollow triple-waved tipped, partial blunt needles with an inner diameter of 0.6 mm (Fig. 1) (Hair Science Institute®, Amsterdam, the Netherlands) under 2x magnification. This needle uses the coarse, dead hairs as a guide and enables us to extract a partially longitudinal follicular unit, even when the follicular unit is not in a perfect triangular configuration.

Figure 1: Close-up of the triple-waved tipped extraction needle.

Fig. 2A shows a follicular unit, containing the visible hairs (brown), hair follicle (dark pink) and connective tissue (white). The needle is placed around the visible hairs and then twisted in the same direction until the grafts are detached from the dermis (Fig. 2A and B). The grafts are extracted with microsurgical forceps (Fig. 2C). The aim of the extraction is to remove only a part of the follicle unit, containing follicle and connective tissue from several hair follicles, and leave sufficient follicle unit tissue behind to regenerate hairs (Fig. 2D and E). After the extraction, Fucidin Cream® (Leo Pharma, Breda, the Netherlands) is applied on the donor area.

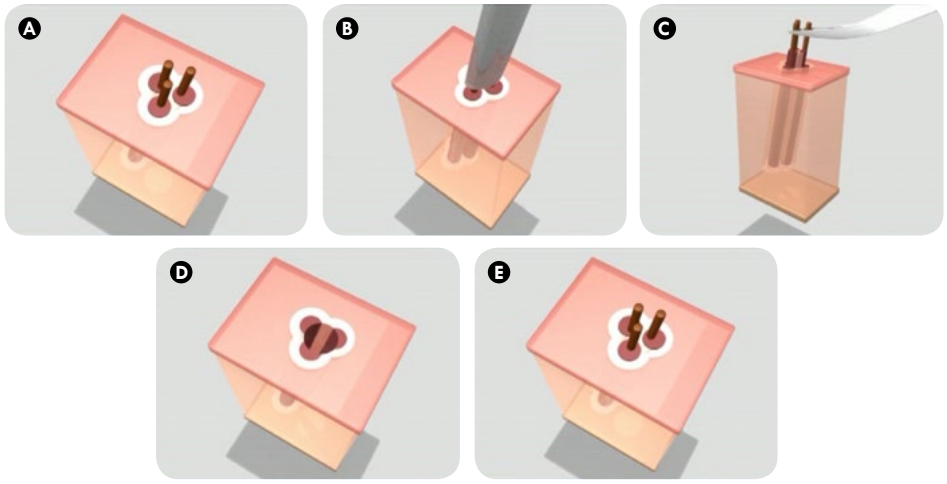

Figure 2: Illustrations of the procedure in the donor area. **(A)** *The whole follicular unit.* **(B)** *Extraction of the longitudinal partial follicular unit with a 0.6 mm needle.* **(C)** *Extraction of the longitudinal partial follicular unit with a micro-surgical forceps.* **(D)** *Part of the follicular unit which is left behind.* **(E)** *Re-growth in the donor area.*

Partial longitudinal follicular units (Fig. 3A and B (Haematoxylin and Eosin (HE) stained transversal sectioned partial longitudinal follicular unit (20x))) are different from complete follicular units (Fig. 3C and D (HE stained transversal sectioned complete follicular unit (10x))), because partial longitudinal follicular units leave a considerable part of the original follicular unit behind at the donor site. They can be distinguished from complete follicular units because partial longitudinal follicular units show terminal hairs at the border and do not contain surrounding tissue and complete follicular units contain complete hair follicles with surrounding tissue.

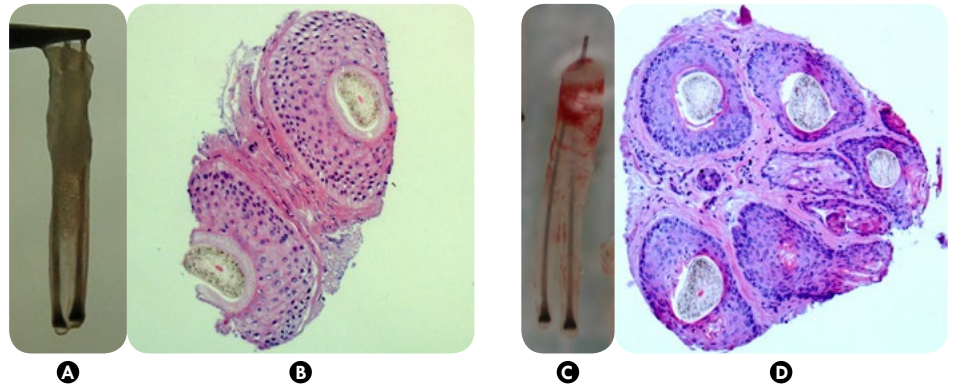

Figure 3: The grafts. **(A)** *Partial longitudinal follicular unit grafts.* **(B)** *Haematoxylin and Eosin (HE) stained transversal sectioned partial longitudinal follicular unit (20x), where a considerable part of the follicular unit is left behind at the donor site.* **(C)** *Complete follicular unit grafts.* **(D)** *Haematoxylin and Eosin (HE) stained transversal sectioned complete follicular unit (10x).*

After extraction, the partial longitudinal follicular units were stored in the preservative medium, which is composed of the following ingredients: sodium chloride, potassium chloride, magnesium sulphate, sodium phosphate, calcium chloride, glucose, sodium bicarbonate, sodium lactate, sodium pyruvate, human serum albumin, insulin, bis(maltolato)oxovanadium (BMOV) and a-tocopherol (vitamin E) (Hair Science Institute®, Amsterdam, the Netherlands) for 2 h until implantation.

1.2.3. Preparation of the recipient area

The recipient area was disinfected with chloorhexidine 2% lotion and anaesthetised with Prilocaïnehydrochloride 30 mg ml^{-1} (3%) and felypressine 0.54 mg ml^{-1} (Citanest) (AstraZeneca). Miniscule holes were made with a hollow needle with an inner diameter of 0.6 mm needle (Hair Science Institute®, Amsterdam, the Netherlands) and skin was removed or the grafts were implanted directly after making the holes with a 21G hypodermal.

1.2.4. Implantation of the grafts

After the preparation of the recipient area, the grafts were implanted with microsurgical forceps.

1.2.5. Follow-up

At intervals of 1 week, and 3 and 12 months after the extraction, the donor area as well as the recipient area was photographed.

RESULTS

2.1 The donor area

Fig. 4 shows the donor area of a patient before (Fig. 4A), and 3 years later after four treatments and 3858 grafts extracted (Fig. 4B). After evaluation of the donor area, there are a few little white spots visible, but almost all hair follicles in the donor site reproduce hairs after 3 years.

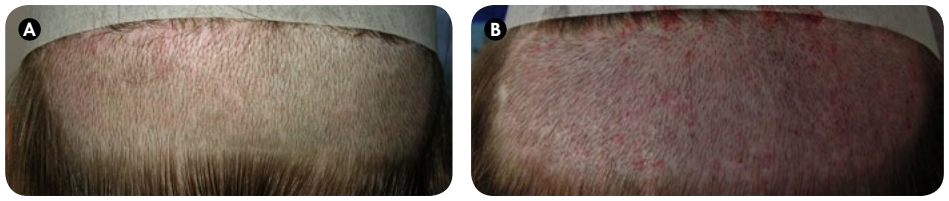

Figure 4: Pictures of the donor area. **(A)** *Picture of the donor area of a patient before harvesting the grafts.* **(B)** *Picture of the donor area of a patient after three years (4 treatments, 3858 grafts extracted).*

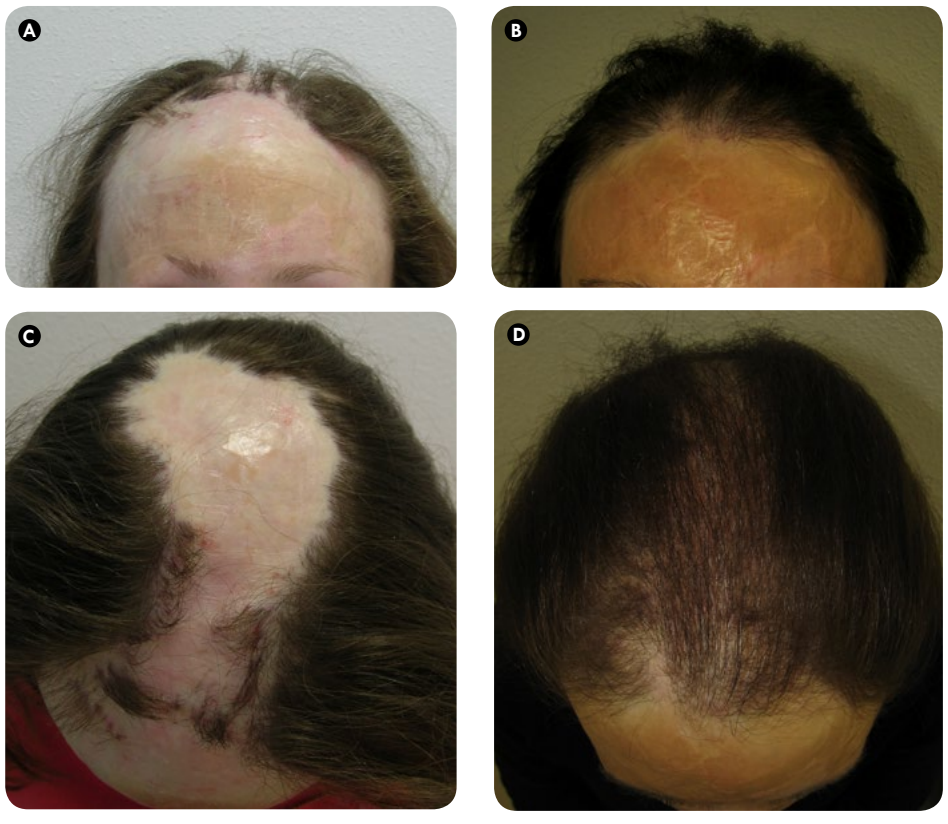

Figure 5: Case 1, KZ after 4 treatments, 3858 grafts. **(A)** *Frontal aspect, before treatment.* **(B)** *Frontal aspect, after four years.* **(C)** *Top view, before treatment.* **(C)** *Top view, after four years.*

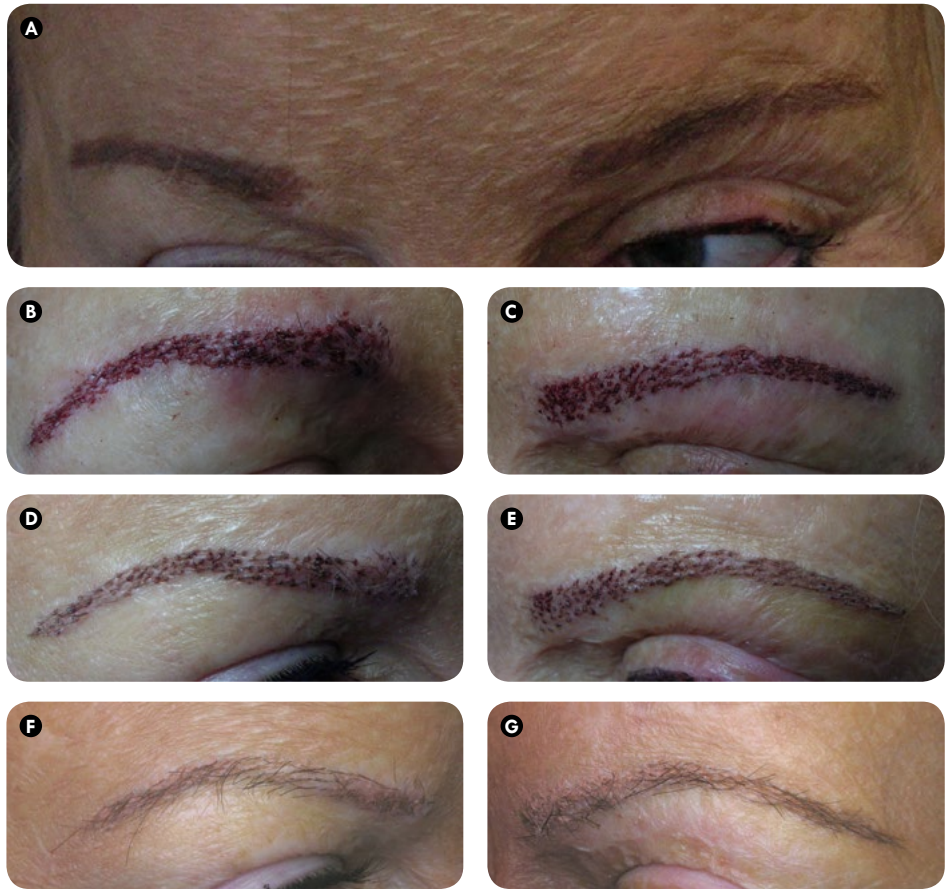

Figure 6: Case 2, MS after 1 treatment, 300 grafts. **(A)** *Before treatment.* **(B)** *Right eyebrow, directly after treatment.* **(C)** *Left eyebrow, directly after treatment.* **(D)** *Right eyebrow, 1 week after treatment.* **(E)** *Left eyebrow, 1 week after treatment.* **(F)** *Right eyebrow, 2 months after treatment.* **(G)** *Left eyebrow, 2 months after treatment.*

2.2 Re-growth of the hairs in the recipient area

Fig. 5 shows the first case (KZ, 23 years), who had, in total, 3858 grafts spread over four consecutive treatments on the scalp and eyebrows during a period of 3 years. The eyebrows were fully restored and the scalp is almost completely covered. She was very satisfied with the result.

Fig. 6 shows the second case (MS, 24 years), who had in total 300 grafts in one session to restore the eyebrows. The eyebrows have been fully restored. Because the eyebrows are very important for the expression of the face, she was very satisfied with the result.

Fig. 7 shows the third case (HW, 40 years), who had, in total, 300 grafts in one session to restore the temple. The temple has been fully restored and she was very satisfied with the result.

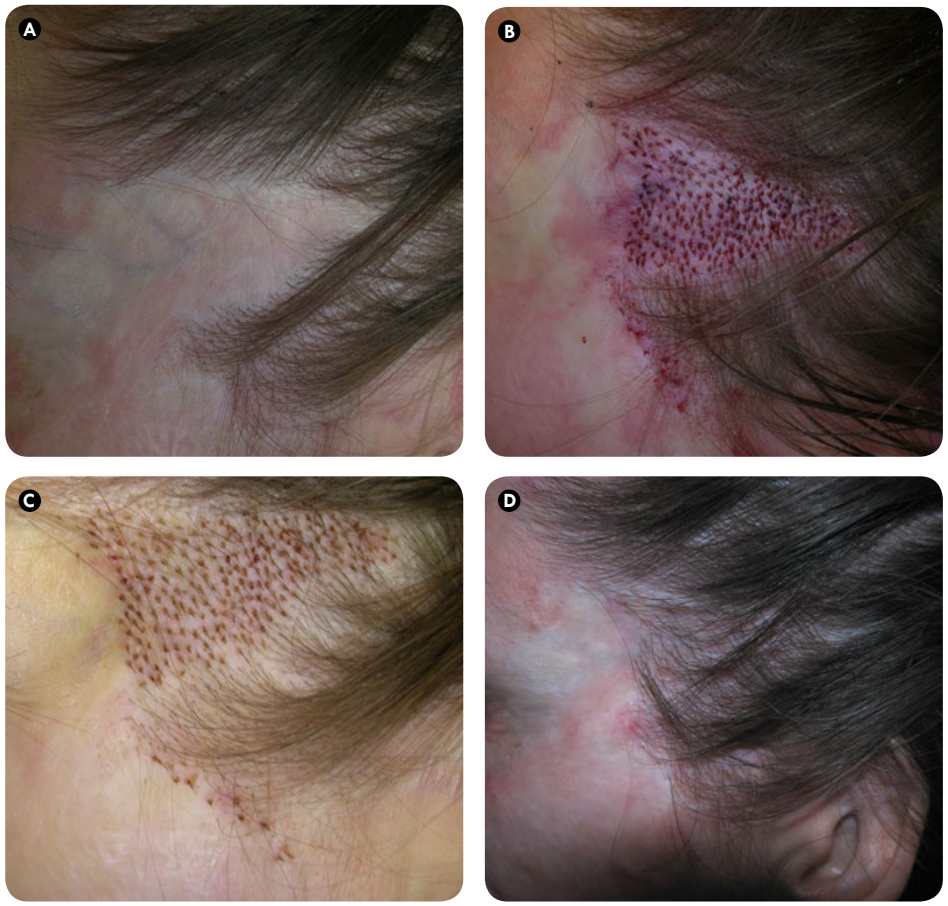

Figure 7: Case 3, HW after 1 treatment, 300 grafts.

(A) Left temporal scalp, before treatment.

(B) Left temporal scalp, directly after treatment.

(C) Left temporal scalp, 1 week after treatment.

(D) Left temporal scalp, 8 months after treatment.

Fig. 8 shows the fourth case (BT, 29 years), who had, in total, 600 grafts in one session to restore the beard area. Consecutive treatments will be necessary to increase the density in the beard. However, as the first session already camouflaged the most visible scars in the area, he was already satisfied after the first treatment.

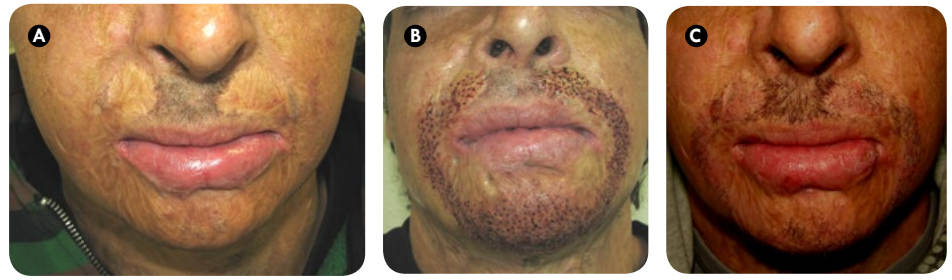

Figure 8: Case 4: BT after 1 treatment, 600 grafts.

(A) Beard, before treatment.

(B) Beard, directly after treatment.

(C) Beard, 1 year after treatment.

Satisfactory cosmetic results are defined as a natural result with a density which covers the scalp. Because these objectives are achieved, all treated patients were satisfied or very satisfied with the results.

DISCUSSION

Although hair transplantation is the best option for facial burn scars, it is really a challenge for the surgeon, because scar tissue behaves differently from normal skin.

In contrast to traditional hair transplantation techniques, which require a strip removal with a depth of 1–1.5 cm to obtain the hair follicles, we showed that successful transplantation in burn scars is feasible using longitudinal partial follicular units with a diameter of 0.5–0.6 mm, and 5–6 mm in length. Due to minimal skin and tissue removal, in the above-mentioned cases, there was minimal to no scarring, pain or other post-surgical trauma such as nerve and vascular damage. In contrast with the traditional hair-transplantation techniques, there is no linear scar, and, therefore, it is impossible that the linear scar will 'stretch out' over time. Further, no stitches or bandages are required.

Potential risks could be density loss of the donor area when the grafts are extracted to close to each other, failure of growth of the implanted PL-FUT due to bad vascularisation as well as necroses when the grafts are implanted too close to each other. However, none of these occurred in the abovementioned cases.

This study shows that partial longitudinal follicular units containing viable follicular stem cells with connective tissue can, even with the complicated tissue characteristics of the recipient site,be used for restoration of the facial hair as well as the scalp in burn victims. Because the partial follicular units, which remain in the dermis in the donor area can survive and re-produce hairs, these follicular units in the donor area can be used again in consecutive treatments. However, more experience with the PL-FUT in burn victims is necessary to evaluate the limitations and further possibilities of this technique.

The main difference between the technique of PL-FUT compared with the other hair-transplantation techniques is the preservation of the donor hair follicles without scarring. As, often in burn victims, this donor area is limited, this aspect is very important. Further, this is especially true for this group of patients.

PL-FUT is a very labour-intensive procedure and to transplant sufficient grafts takes a full day. However, this technique may represent the first reliable patient-friendly method to generate two hair follicles from one hair follicle with consistent results and preservation of the donor area. Therefore, this method is very suitable for people with very limited donor area, the most extreme example of this being burn victims. All of the presented patients in this article got reimbursement from their health insurance companies.

In general, we prefer the improved hair transplantation method PL-FUT above all other surgical possibilities for hair restoration such as traditional hair transplantation techniques (strip-method or FUE).

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4.3 RESTORATION OF THE EYEBROWS BY HAIR TRANSPLANTATION

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Gho, C. and M. Neumann (2014). "Restoration of the eyebrows by hair transplantation." Facial Plast Surg 30(2): 214-218.

ABSTRACT

Partial loss of the eyebrows can be the result of epilation, scars, and inflammatory diseases. Facial hair and eyebrows play a major role in our mimetic expression and interaction. Therefore, facial hair restoration of the eyebrows can improve the appearance and psychological well-being of patients. We report the use of partial longitudinal follicular unit transplantation (PL-FUT) to restore eyebrows. A total of 10 patients (age between 18 and 59 years; mean, 39 years) have been treated with PL-FUT to restore the eyebrows. The grafts were harvested from the occipital area of the scalp. Suitable grafts were impregnated with a preservative solution and implanted into the eyebrows areas. Hair growth in the donor area as well as the eyebrows was observed before treatment and at intervals of 1 week, 3 months, and 1 year after treatment. Evaluation of the donor area reveals no visible scars with almost all hair follicles in the donor site reproducing hairs after 1 year. All treated patients were satisfied or very satisfied with their cosmetic results. PL-FUT is a reliable, patient friendly method suitable for hair restoration of eyebrows in healthy persons as well as in burn scar tissue.

INTRODUCTION

Loss of the hairs of the eyebrows is not uncommon. Inflam-matory skin diseases such as Lupus Erythematosus[1] or hypo-thyroidism[2] can cause partial loss of the eyebrows, but most partial loss of the eyebrows is caused by epilation or scars.[3] Our facial appearance plays a central role in the psychological well-being and self-esteem of the individual. Facial hair plays a major role in our mimetic expression and interaction. While partially framing the eyes, the eyebrows transmit expressions and emotions and communicate feelings.[4,5] Facial hair restoration including eyebrows, beard, and scalp can improve the appearance and psychological well-being of humans.

There are different hair transplantation techniques used for the restoration of eyebrows. The most commonly used hair transplantation methods are the so called "strip" method and the follicle unit extraction (FUE) method.[6,7] Disadvantages of these techniques include the size of the grafts which can result in lower density and unnatural results. Previous re-search revealed that both proximal and distal areas of the hair follicle contain follicular stem cells that can induce hair growth.[8]

Partial longitudinal follicular unit transplantation (PL-FUT) is an innovative hair transplantation technique where partial follicular units are extracted in a longitudinal fashion with a diameter of 0.5 to 0.6 mm and 5 to 6 mm in length. With this technique follicular stem cells remain at the donor site and in the extracted partial follicle. In this case, two hair follicles can be generated from one. As the size of the grafts is much smaller than the conventional techniques a higher density can be achieved.

PL-FUT is a very reliable, patient friendly method to gener-ate two hair follicles from one hair follicle with consistent results and preservation of the donor area.[9] This technique has worked with excellent results and patient satisfaction for androgenic alopecia and burn victim facial hair restoration in men and women.[10] We report the use of PL-FUT to restore eyebrows.

PATIENTS AND METHODS

Patients

A total of 10 patients (age between 18 and 59 years; mean, 39 years) consulted Hair Science Institute with sparse or absent eyebrows from different causes. They were all treated with PL-FUT to restore the eyebrows (Table 1). In this article, we evaluate these patients and their results.

Case	Initials (gender)	Date of birth (age)	Treatment	Treatment date	Diagnosis	Number of grafts
1	**MB** (F)	Jan 22, 1959 (52)	Total restoration	Jun 28, 2011	Because of epilation	350
2	**MB** (F)	Mar 1, 1967 (44)	Total restoration	Dec 13, 2011	Because of epilation	236
3	**SG** (M)	Apr 2, 1993 (18)	Partial restoration	Feb 23, 2012	Burn scars	250
4	**FdeG** (M)	Mar 9, 1961 (58)	Total restoration	Mar 1, 2012	Burn scars	291
5	**MI** (F)	Dec 9, 1960 (50)	Partial restoration	May 3, 2011	Natural sparse eyebrows/epilation	300
6	**KJ** (F)	Feb 2, 1973 (37)	Partial restoration	May 3, 2010	Because of epilation	300
7	**JM** (F)	Nov 10, 1950 (59)	Total restoration	Jun 13, 2010	Frontal fibrosing alopecia	300
8	**MS** (F)	Mar 4, 1975 (35)	Total restoration	Nov 19, 2010	Because of epilation	300
9	**MS** (F)	Apr 29, 1986 (19)	Total restoration	Apr 24, 2006	Burn scars	300
10	**KZ** (F)	Aug 2, 1986 (18)	Partial restoration	Jun 30, 2005	Burn scars	250

Table 1: Patients

LONGITUDINAL PARTIAL FOLLICULAR UNIT TRANSPLANTATION[9]

Preparation of the Donor Site

On the occipital side of the scalp, an area of 10 x 4 cm was shaved, disinfected with Chlorhexidine 2% lotion, and anaes-thetized with Lidocaine 2% with 1:100,000 epinephrine (AstraZeneca, Monts, France).

Extraction of the Partial Longitudinal Follicular Units (Grafts)

The grafts were harvested with hollow triple-waved tipped, partial blunt needles with an inner diameter of 0.6 mm (Hair Science Institute, Amsterdam, The Netherlands). After the extraction, Fucidin cream (Leo Pharma, Breda, The Netherlands) was applied on the donor area. After extraction, the partial longitudinal follicular units were stored in the preservative solution (Hair Science Institute, Amsterdam, The Netherlands) for 2 hours until implantation.

Design of the Eyebrow

The eyebrows were designed using the following method. First, the lateral point of the eyebrow goes from the lateral nasal ala, through the lateral canthus (Fig. 1A). Second, the highest point of the eyebrows goes from the lateral nasal ala, through the middle of the pupil (Fig. 1B). Finally, the most medial side of the eyebrow can be drawn from the lateral nasal ala through the medial canthus (Fig. 1C).

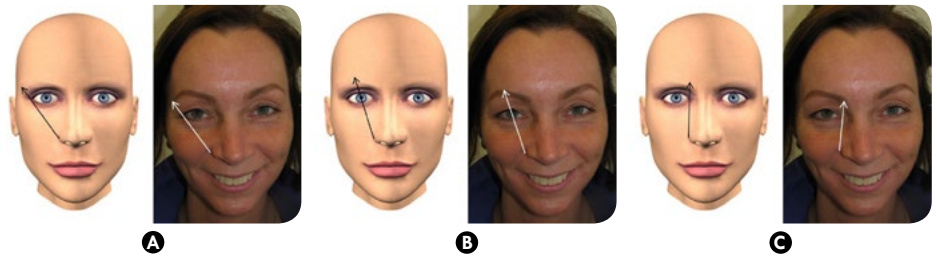

Figure 1: Design of the eyebrow. **(A)** *First, the lateral point of the eyebrow goes from the lateral nasal ala, through the lateral canthus.* **(B)** *The highest point of the eyebrows goes from the lateral nasal ala, through the middle of the pupil.* **(C)** *The most medial side of the eyebrow can be drawn from the lateral nasal ala, through the medial canthus.*

Preparation of the Recipient Area

After designing of the new eyebrows, the recipient area was disinfected with Chlorhexidine 2% lotion and anaesthetized with Prilocaïnehydrochloride 30 mg/mL (3%) and felypressine 0.54 µg/mL (Citanest) (AstraZeneca).

Implantation of the Grafts

The grafts were implanted with microsurgical forceps directly after making the recipient holes with a 21 G (0.723 mm) hypodermal Luer lock needle. The direction as well as the angle of the implantation is very important to get a natural result. The direction varies from caudal-medial to cranial at the medial side of the eyebrow to medial to lateral at the lateral side from the eyebrow (Fig. 2A). As the eyebrow is three dimensional, the hairs in the border of the eyebrows are implanted like a fishbone (Fig. 2B). The angle of implantation should be as flat as possible (Fig. 2C).

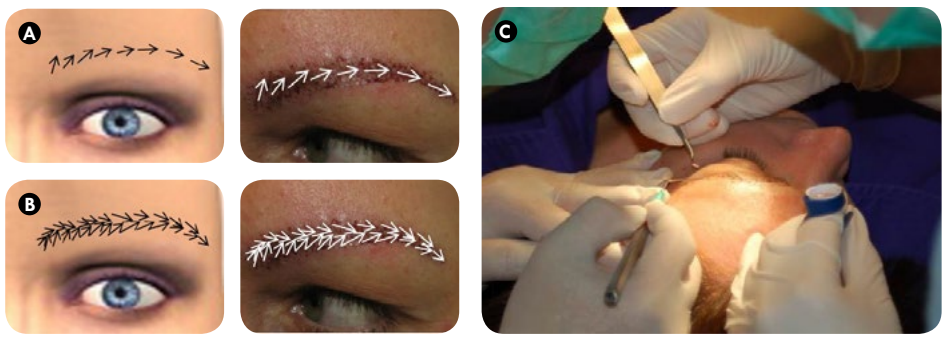

Figure 3: The donor area. **(A)** *Donor area, before treatment.* **(B)** *Donor area, after 1 year (300 grafts).*

4.3

Follow-up

At intervals 1 week, 3 months, and 1 year after the PL-FUT, the donor areas and the recipient areas were photographed

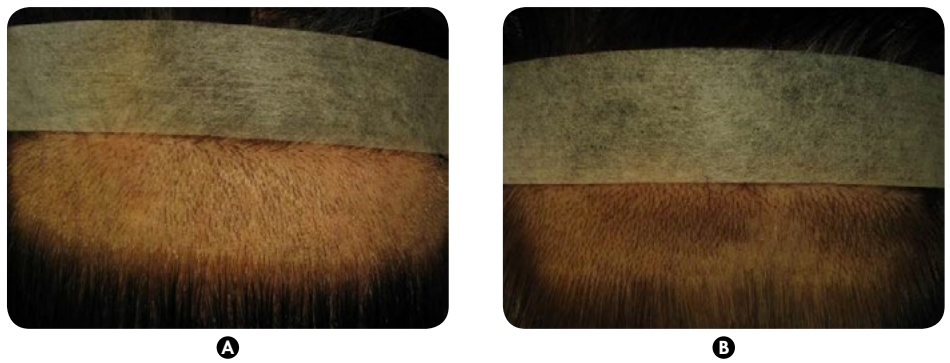

Figure 2: Implantation of the grafts. **(A)** *The direction of the implantation of the grafts is very important and varies from caudal-medial to cranial at the medial side of the eyebrow to medial to lateral at the lateral side from the eyebrow.* **(B)** *As the eyebrow is three dimensional, the hairs in the borders of the eyebrows are implanted like a fishbone.* **(C)** *The angle of implantation has to be as flat as possible.*

RESULTS

The Donor Area

Fig. 3 shows the donor area of a patient before (Fig. 3A) and 1 year after treatmen with 300 grafts extracted (Fig. 3B). Evaluation of the donor area reveals no visible scars, and almost all hair follicles in the donor site reproduced hairs after extraction.

Restoration of the Eyebrows:
Case 4, FdeG: 291 Grafts

Fig. 4 shows the fourth case (FdeG, male, 58 years), who had 291 grafts in the eyebrows. The eyebrows have been fully restored. Because of burn scars, there are still little gaps in the eyebrows. The patient is very satisfied with the results.

Case 6, KJ: 300 Grafts

Fig. 5 shows the sixth case (KJ, female, 37 years), with a total of 300 grafts in one session to restore the eyebrows. The eyebrows have been fully restored. She is very satisfied with her results stressing the importance of the eyebrows in facial expression.

Satisfactory cosmetic results are defined as natural results and natural density of the eyebrows. With these objectives achieved, all treated patients were satisfied or very satisfied with their results.

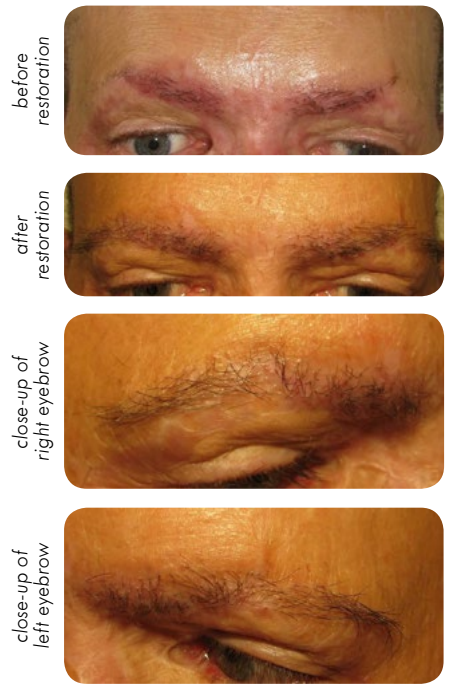

Figure 4: FdeG, 291 grafts.

Figure 5: KJ, 300 grafts.

4.3

DISCUSSION

There are many options to mimic missing eyebrows including permanent makeup. However, permanent makeup appears unnatural mostly because this technique only restores the eyebrows in two dimensions. Permanent makeup for the eyebrows will also fade with time, change colors and can develop contact dermatitis against the dye. In our opinion, hair transplantation is the best option to restore the eyebrows in a natural way, especially for burn victims. Burn victim eyebrow restoration is even more challenging for the surgeon because scar tissue behaves differently from normal skin.

To obtain the most natural result as possible, the surgeon must concentrate on the shape and hair characteristics of the eyebrow. This design makes the difference between new natural eyebrows or just a stripe of hair. Restoration of the eyebrows is significantly different compared with the restoration of the scalp because of the precise angle, direction, and symmetry between the left and right eyebrows as well as the density of implantation. Also, the transplantation should be initially done in a cosmetically perfect way, because improve-ment or touch-up procedures afterward are undesirable and nearly impossible. Therefore, special education for hair restoration of eyebrows is desirable.

We showed that successful restoration of natural looking eyebrows is feasible with PL-FUT, using longitudinal partial follicular units with a diameter of 0.5 to 0.6 mm and 5 to 6 mm in length, even in burn scars.[10]

These patients did not experience any adverse effects after the treatment. Because of minimal skin and tissue removal during harvesting of the grafts, and the use of 21 G (0.723 mm.) needles for the implantation of the grafts, there is minimal to no scarring, pain, or other postsurgical trauma such as nerve and vascular damage.

Because the diameter of grafts is much smaller compared with the grafts of conventional techniques such as the strip method or FUE method, we can achieve almost the same density as a normal eyebrow, which is approximately 50 hairs/cm^2.

However, there are some limitations and disadvantages with PL-FUT. The skin of burn scars behave differently. In these cases, the direction of the existing as well as the implanted hairs can change in time, as the skin has changed.

As the donor follicles are derived from the occipital side of the scalp, the extracted partial longitudinal follicular units transplanted have the same characteristic as the donor site. The structure, color, as well as the hair growth pattern of the transplanted hairs are different than the original hairs from the eyebrow. Although the transplanted hairs might be coarser and have color differences than the original eyebrows, in practice, these differences have little effect on the natural aspect of the eyebrows. The hairs will grow as long as the hairs in the donor area and may need occasional trimming.

This study shows that partial longitudinal follicular units containing viable follicular stem cells with connective tissue transplanted to the recipient area can be used for hair restoration of eyebrows in healthy persons as well as in burn scar tissue.[10]

The main difference between the technique of PL-FUT and other hair transplantation techniques is the size of the extracted partial longitudinal follicular units. As these grafts are considerable, smaller compared with the grafts obtained by the strip method and FUE method, the precision, density, angle, and direction of the eyebrows are better.

PL-FUT is a very labor intensive procedure to restore eye-brows and takes a full day. Because eyebrows play a central role in the psychological as well as physical self-esteem of the individual, restoration of the eyebrows with PL-FUT is preferable rather than traditional hair transplantation techniques such as the strip method or FUE method.

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4.4 7 YEARS' EXPERIENCE WITH PARTIAL LONGITUDINAL - FOLLICULAR UNIT TRANSPLANTATION (PL-FUT)

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ABSTRACT

Background
Partial Longitudinal - Follicular Unit Transplantation (PL-FUT) is a hair restoration technique for androgenic as well as cicatricial alopecia allowing to multiply hair follicles in vivo with preserving the donor area.

Objectives
To evaluate the effectiveness of PL-FUT.

Materials & Methods
A retrospective single center study between 2005 to 2011. The number of treatments, satisfaction rate, side effects and regrowth on donor and recipient area, average number of grafts were evaluated after one week and one year.

Results
4819 hairloss patients visits; 1983 (41.1%) patients have been treated with 2646 treatments in total, 1983 (74.9%) initial and 663 (25.1%) consecutive treatments were performed. 2306 (87.1%) treatments have been evaluated of which 2202 (95.5%) of the evaluated treatments were considered satisfactory according to the patients after one year.

The average number of grafts increased from 635 grafts in 2005 to 1159 grafts per treatment session in 2011. In the donor area, no visible density loss in 2276 (98.7%) and some density loss in 30 (1.3%) patients was observed.

Conclusions
PL-FUT has been shown an effective technique for androgenic and cicatricial alopecia with a high patients satisfaction, minimal side effects, natural result and preservation of the donor area.

INTRODUCTION

The scalp and face plays a central role in the psychological and physical self-esteem of the individual. It transmits expressions and emotions, communicates feelings and allows for individual identity.[1,2] Although this depends on the sex, age and sociocultural background, baldness has a negative effect on the self-esteem.[3] Besides the psychological impact of baldness, recent scientific research has shown that the bald scalp is more exposed to sunlight, and therefore leads to early aging of the skin, including actinic keratosis.[4] Hair restoration treatments contributes for the physical as well as physiological wellbeing of the patient.

The ideal hair transplantation should have an excellent cosmetic outcome, 100% hair re-growth, 100% preservation and therefore endless source of donor follicles, no scarring, safe, an easy procedure with a short treatment duration, no down time and inexpensive. Of all currently available techniques, there is no technique which fulfills all mentioned criteria. All techniques have their advantages and disadvantages. The most known hair transplantation method is the so called "strip" method.[5] A strip of skin containing hair follicles is removed (leaving a large linear scar), cut into grafts and implanted in the recipient area. The Follicle Unit Extraction (FUE) method is very promising in which whole follicle units are extracted one by one and implanted into the recipient area.[6] Although the FUE method is more patient friendly and leaves pinpoint scars, the major disadvantage of both methods is that the extracted hair follicles are removed in total and reducing density at the donor side. For maximal preservation of donor follicles, the Partial Longitudinal - Follicular Unit Transplantation (PL-FUT)[1] technique has been developed.[7]

We hereby reveal our experiences with PL-FUT.

MATERIALS AND METHODS

Patients

Retrospectively from 2005 to 2011, patients who visited Hair Science Institute for consultation were actively registered. A distinction is made between patients who had their first treatment and patients who had a consecutive treatment.

Partial Longitudinal Follicular Unit Transplantation (PL-FUT)

PL-FUT was performed as described earlier.[7] In brief, grafts were harvested with hollow wave-tipped needles with an inner diameter of 0.6 mm from the occipital area of the scalp. The suitable longitudinal partial follicular units (grafts) were impregnated with a preservative medium[8], and implanted into the recipient area. The number of transplanted hairs were counted.

Post-treatment evaluation

All patients were invited via telephone or email to personally evaluate their treatment one week and one year after treatment. The objective evaluations have been performed by an experienced nurse or physician of Hair Science Institute.

One week follow-up of PL-FUT.

All patients treated with PL-FUT were asked to score the extent of their experienced pain, numbness, itching, swelling and bleeding on a 5 point scale for both the donor as well as recipient site (none (0), little bit (-), moderate (±) and (very) much (+/++)).

One week after the PL-FUT, a physician or nurse scored the extent of infection, erythema, crusts and swelling at both sites on a 5 point scale (none (0), almost none (--), little bit (-), moderate (±) and (very) much (+/++)). In addition, pictures of the donor area as well as the recipient area were taken.

One year follow-up of PL-FUT.

The self-reported overall patient satisfaction was evaluated in the categories not satisfied (0), partly satisfied (±) and satisfied/very satisfied (+/++).

At the donor area, the hair density was scored by a physician or nurse: 'no-regrowth', 'density decreased' and 'normal density'.

At the recipient area, re-growth in the was evaluated by a physician or nurse: less than 25%, 26-50%, 51-75% and more than 75%. In addition, pictures of the donor area and the recipient area were taken.

Statistical Analysis

No statistical analysis have been performed.

Year	Number of information requests	Consultations (age)	Total number of treatments	Patients decided to perform treatment	Consecutive treatments	Average age	Male	Female
	a	b	c	d	e	f	g	h
2005	70	63	26	26	0	42.04	22	4
2006	340	282	212	198	14	42.07	171	27
2007	418	372	316	259	57	43.49	198	61
2008	677	613	378	300	78	43.09	242	58
2009	758	625	395	267	128	43.76	189	78
2010	1091	926	625	457	168	42.63	333	124
2011	1465	1281	694	476	218	43.62	361	115
Total	4819	4162	2646	1983	663	43.18	1516	467

Table 1: Table 1. Patient data & treatments

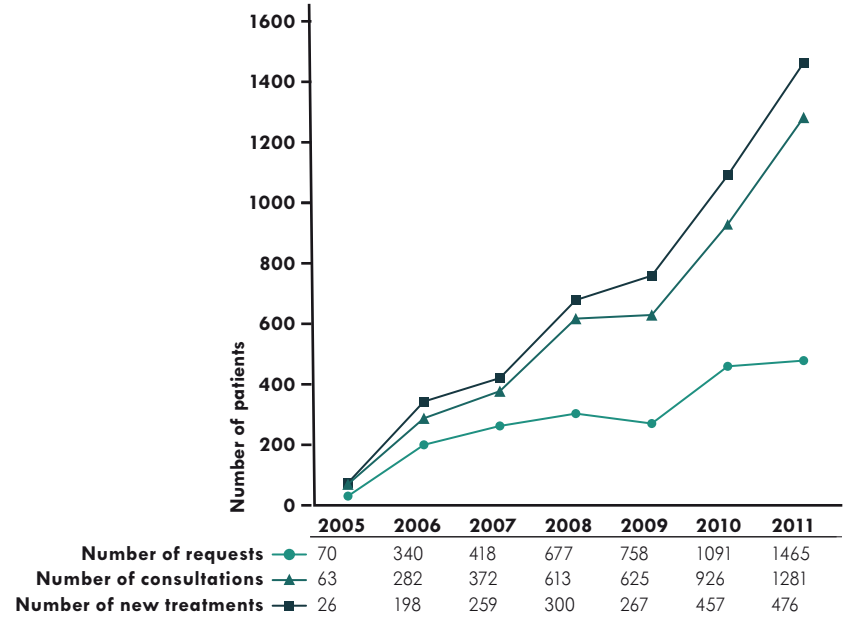

Figure 1: Number of patients per year

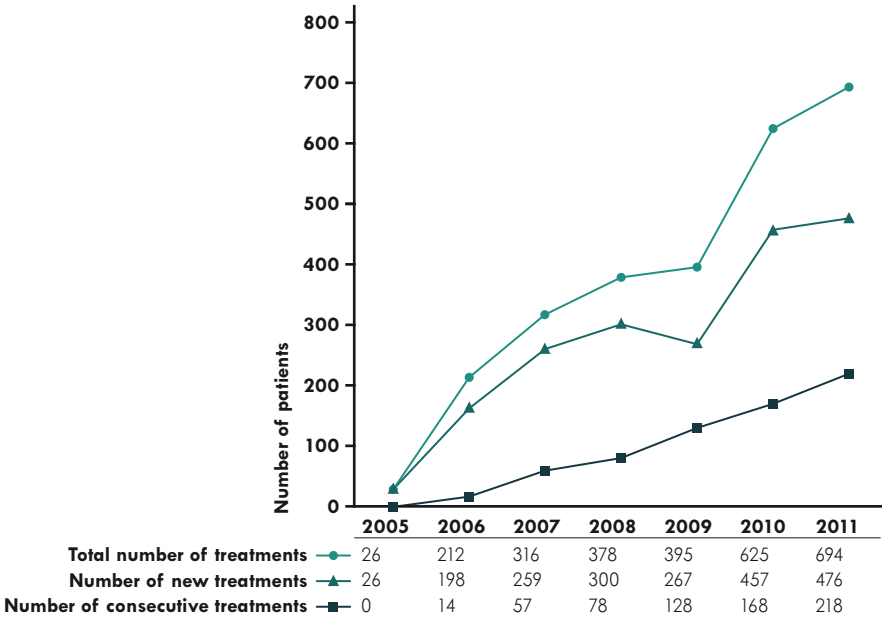

Figure 2: Number of treatments

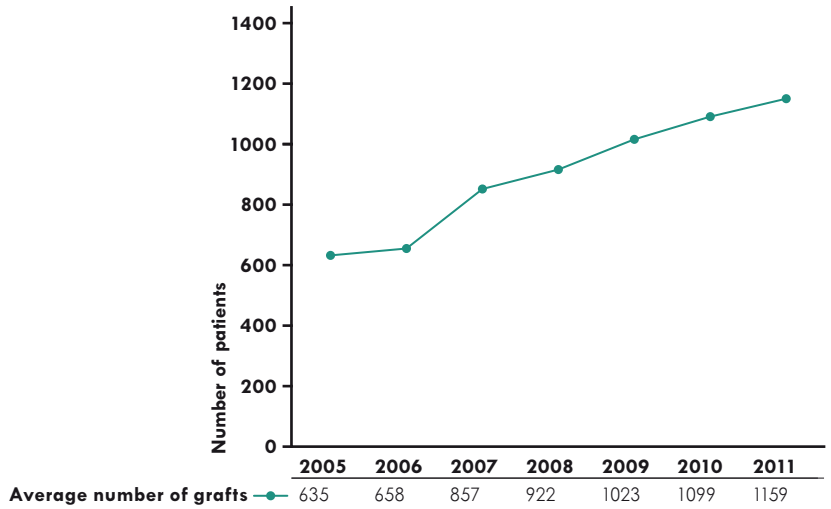

Figure 3: Average number of grafts

RESULTS

Patients

Between 2005 – 2011, 4819 patients visited Hair Science Institute. The number increased from 70 in 2005 to 1465 patients in 2011 (Table 1 & Figure 1). The average age of 1983 patients at their initial PL-FUT was 43 years and 1516 (76%) were male patients (Table 1).

Treatments

A total of 2646 PL-FUTs were performed. From the 2646, 1983 (74.9%) were initial and 663 (25.1%) were consecutive PL-FUTs. The proportion who underwent a consecutive treatment increased from 7.1 % in 2006 to 45.8% in 2011 (Table 1 & Figure 2).

Number of grafts per day (Figure 3)

The average number of grafts implanted from the 2646 treatments was 908 per day. The number of implanted grafts increased from 635 in 2005 to 1159 in 2011 (Table 2 & Figure 3).

One week follow-up of PL-FUT (Table 2 & 3)

At the donor site, the most common adverse events were significant pain (+/++) 4.1%, numbness (+/++) in 6 cases (0.2%), significant itching (+/++) in 219 cases (8.3%), significant swelling (+/++) in 15 cases (0.6%) and significant bleeding in none of the donor sites following PL-FUTs (0%).

At the recipient site, significant pain (+/++) in 17 cases (0.64%), numbness (+/++) in 16 cases (1.28%), significant itching (+/++) in 56 cases (2.12%), significant swelling (+/++) in 165 cases (6.24%) and significant bleeding (+/++) in 3 cases (0.11%) following the 2646 PL-FUT treatments.

At physical examination of the donor area one week after treatment, severe infection was experienced in 0 cases, severe erythema was experienced in 4 cases (0.2%), significant crust formation in 21 cases (0.8%) and significant swelling in 3 cases (0.1%).

At physical examination of the recipient area, severe infection was experienced in 0 cases (0%), severe erythema was experienced in only 1 case (0.04%), significant crust formation in 169 cases (6.4%) and significant swelling in 11 cases (0.4%).

Year	Number of treatments	Pain					Numbness					Itching					Swelling					Bleeding				
		0	-	-+	+	++	0	-	-+	+	++	0	-	-+	+	++	0	-	-+	+	++	0	-	-+	+	++
2005	26	15	5	5	0	1	22	4	0	0	0	7	5	6	6	1	23	2	0	0	1	26	0	0	0	0
2006	212	152	34	16	10	0	201	6	4	1	9	115	42	29	21	5	202	5	3	2	0	211	1	0	0	0
2007	316	229	56	20	11	0	299	12	3	2	0	180	63	44	22	7	289	20	3	2	2	308	0	8	0	0
2008	378	290	47	27	11	3	349	26	2	1	0	225	72	44	30	7	353	17	5	3	0	371	0	7	0	0
2009	395	300	55	21	15	4	367	27	0	1	0	241	82	49	19	4	373	19	2	1	0	379	16	0	0	0
2010	625	476	85	41	22	1	592	26	6	1	0	396	108	78	36	7	596	23	5	1	0	611	14	0	0	0
2011	694	574	31	59	29	1	630	57	7	0	0	381	178	81	47	7	645	36	10	3	0	683	11	0	0	0
Total	2646	2036	313	189	98	10	2460	158	22	6	0	1545	550	331	181	38	2481	122	28	12	3	2589	42	15	0	0
a	b	c					d					e					f					g				

Year	Number of treatments	Pain					Numbness					Itching					Swelling					Bleeding				
		0	-	-+	+	++	0	-	-+	+	++	0	-	-+	+	++	0	-	-+	+	++	0	-	-+	+	++
2005	26	19	7	0	0	0	23	9	4	0	0	15	8	3	0	0	19	2	1	2	2	0	0	1	0	0
2006	212	186	17	6	2	1	150	38	21	3	0	158	42	8	4	0	149	26	16	18	3	209	0	3	0	0
2007	316	284	23	7	2	0	268	32	7	7	2	231	52	26	7	0	220	35	23	28	10	310	0	5	1	0
2008	378	339	29	9	1	0	316	43	17	2	0	322	29	21	4	2	273	48	28	23	6	371	0	6	0	0
2009	395	352	31	9	3	0	249	36	15	4	0	315	54	15	9	2	308	47	24	9	7	389	0	6	0	0
2010	625	543	63	15	4	0	533	58	25	8	1	485	83	45	9	3	487	70	41	19	8	613	0	11	1	0
2011	694	596	82	12	3	1	578	83	26	7	0	504	132	42	15	1	510	102	52	20	10	677	0	17	0	0
Total	2646	2319	252	58	15	2	2208	299	115	31	3	2030	400	160	48	8	1966	330	185	119	46	2569	0	49	3	9
a	b	h					i					j					k					l				

Table 2: Subjective evaluation of the Longitudinal Partial Follicular Unit Transplantation (PL-FUT) after one week

Year	Number of treatments	Inflammation						Erythema						Crusts						Swelling						Regrowth				
		0	-	-+	+	++	+++	0	-	-+	+	++	+++	0	-	-+	+	++	+++	0	-	-+	+	++	+++	0	-+	+	++	+++
2005	26	25	0	1	0	0	0	21	4	1	0	0	0	19	5	1	0	1	0	26	0	0	0	0	0	-	1	0	0	0
2006	212	206	4	1	1	0	0	191	15	6	0	0	0	186	14	9	2	1	0	209	3	0	0	0	0	-	2	0	0	0
2007	316	312	3	1	0	0	0	243	52	17	2	2	0	253	37	16	5	4	1	308	6	0	0	1	1	-	4	2	0	2
2008	378	377	1	0	0	0	0	305	46	24	3	0	0	311	43	14	4	6	0	376	1	1	0	0	0	-	3	2	4	0
2009	395	393	2	0	0	0	0	325	45	17	7	1	0	357	19	11	4	3	1	392	3	0	0	0	0	-	1	1	0	0
2010	625	619	3	2	1	0	0	551	57	14	3	0	0	583	29	8	2	3	0	617	7	0	0	1	0	-	0	0	3	1
2011	694	690	2	2	0	0	0	624	57	9	3	1	0	652	33	4	4	1	0	692	2	0	0	0	0	-	4	2	1	0
Total	2646	2622	15	7	2	0	0	2260	276	88	18	4	0	2361	180	63	21	19	2	2620	22	1	0	2	1	0	15	7	8	3
a	b	c						d						e						f						g				

Year	Number of treatments	Inflammation						Erythema						Crusts						Swelling						Regrowth				
		-	-+	+	++	+++	0	-	-+	+	++	+++	0	-	-+	+	++	+++	0	-	-+	+	++	+++	0	-	-+	+	++	+++
2005	26	0	0	0	0	0	23	3	0	0	0	0	20	4	1	1	0	0	12	3	4	3	3	1	23	2	0	0	0	0
2006	212	0	0	0	0	0	208	3	1	0	0	0	188	16	6	2	0	0	142	15	30	14	10	1	201	9	0	0	0	0
2007	316	0	0	0	0	0	315	0	1	0	0	0	249	57	8	2	0	0	186	32	38	28	24	8	301	7	0	0	0	0
2008	378	0	0	0	0	0	376	2	0	0	0	0	305	55	16	2	0	0	238	33	25	40	26	16	361	8	0	0	0	0
2009	395	0	0	0	0	0	395	0	0	0	0	0	332	44	13	5	1	0	282	24	43	14	24	8	387	6	0	0	0	0
2010	625	0	0	0	0	0	625	0	0	0	0	0	549	59	14	3	0	0	516	41	18	22	21	7	613	8	0	0	0	0
2011	694	0	0	0	0	0	693	1	0	0	0	0	617	63	12	2	0	0	582	32	34	26	10	10	682	5	0	0	0	0
Total	2646	0	0	0	0	0	2635	9	2	0	0	0	2260	298	70	17	1	0	1858	180	192	147	118	51	2568	45	0	0	0	0
a	b	h					i						j						k						l					

Table 3: Objective evaluation of the Longitudinal Partial Follicular Unit Transplantation (PL-FUT) after one week

One year evaluation of PL-FUT (Table 4)
From the 2646 PL-FUTs, 2306 (87.2%) were evaluated after one year. At the donor site, the hair density was decreased in 30 people (1.30%) and stable in the remaining patients. At the recipient site, the distribution of extent of re-growth is demonstrated in Table 5; In 39 of the 2306 treated patients (1.69%), the re-growth in the recipient area was less than 75% according to a physician or nurse.

Satisfaction one year after PL-FUT (Table 5)
During the evaluation period, 2202 of the 2306 patients; (95.5%) were (very) satisfied. There was no significant difference in satisfaction between the PL-FUTs performed during the 7 years evaluation period (data not shown).

				Result - Satisfaction				
a	b	c	d	e				
Year	**Number of treatments**	**Non-responders**	**Responders**	**Not satisfied**	**Party satisfied**	**Satisfied**	**Very satisfied**	**Very satisfied**
2005	26	2	24	2	0	8	14	22
2006	212	14	198	0	2	121	75	196
2007	316	40	276	6	14	138	118	256
2008	378	53	325	10	9	168	138	306
2009	395	67	328	1	5	177	145	322
2010	625	89	536	7	16	292	221	513
2011	694	75	619	6	26	358	229	587
Total	2646	340	2306	32	72	1262	940	2202

Table 5: Satisfaction 1 year after Longitudinal Partial Follicular Unit Transplantation (PL-FUT)

a	b	c	d	e	f					
				Donor area Regrowth	**Recipient area Re-growth**					
Year	**Number of treatments**	**Non-responders**	**Responders**	**No re-growth**	**Density Decreased**	**Normal Density**	**0**	**0-25**	**25-50**	**50-75**	**75-100**	**100**	**75-100**
2005	26	2	24	0	0	24	0	1	0	0	18	5	23
2006	212	14	198	0	1	197	0	0	0	18	108	72	180
2007	316	40	276	0	4	272	0	0	2	1	158	115	273
2008	378	53	325	0	2	323	0	0	1	2	190	132	322
2009	395	67	328	0	3	325	0	0	0	1	154	173	327
2010	625	89	536	0	7	529	0	0	1	3	477	55	532
2011	694	75	619	0	13	606	0	2	2	5	512	98	610
Total	2646	340	2306	0	30	2276	0	3	6	30	1617	650	2267

Table 4: 1 Year evaluation of the Longitudinal Partial Follicular Unit Transplantation (PL-FUT)

DISCUSSION

Alopecia is a serious problem for the majority of men and especially women. Hair transplantation is up to now the best technique for hair restoration.[9] There are different hair transplantation techniques from Strip to the FUE method. The FUE technique is the most popular.[10] The main difference between the PL-FUT technique compared to the classic techniques[5,6] is the preservation of at least a part of the donor hair follicles in combination with a very limited scarring. In general, no long term follow-up studies are available for hair restoration. We have evaluated our 7 years' experience with the PL-FUT.

In this 7 years period, we performed 2646 PL-FUT procedures, with an average self-reported patient satisfaction rate of 95,5%. The number of treated patients grew, but the percentage of patients who decided to be treated decreased. The relative decrease of treated patients is probably due to the recession in Europe started in 2008. Another explanation could be that more marketing leads to less motivated patients who asked for a free of charge consultation. The percentage of male patients did not vary significantly (2007: 198 of the 259 patients; 76.4%, and 2011: 361 of the 476; 75.8%) compared to the total amount of patients, possibly due to the higher prevalence of androgenetic alopecia (AGA) in men (50% at 50 years of age) compared to women (30% at 50 years of age) of the Caucasian ethnicity[11] and also the knowledge that women can have good results from hair transplantation is less present in the society.

Severe complications like infection and swelling can be expected shortly after transplantation and cosmetic results are only fully visible after one year. For this, all treated patients have been evaluated one week as well as one year after transplantation. Comparison between the first and last year of treatment results has not showed important differences at all scored criteria (table 1 to 6). In comparison with the traditional hair transplantation techniques, the PL-FUT has even after a second intervention nearly no visible scars at the donor site, resulting in a much better cosmetic preservation of the donor area. This also explains that a second intervention is a relatively easy option to increase the density at the desired recipient area which is important to obtain an optimal natural appearance. The PL-FUT has it risks and limitations, which are partly the same as the traditional FUE, but some are specific for this technique.

Smaller grafts are more vulnerable and have less metabolic reserve to survive the time between extraction and implantation. The grafts are stored for in a preservation solution in between extraction and implantation, which contains Bis(maltolato)oxovanadium.[12] This solution has proven to preserve nearly all grafts ex vivo and is by far superior than saline solution.[8]

The final and cosmetic result depends not only on the grafts type (single-hair grafts or follicular units), the survival rate of the transplantation and the skill of the surgeon, especially how and where the grafts are placed, but also on the number of grafts one can transplant. The human factor is one of the major factors for variability of any (cosmetic) procedure, and is therefore also a common problem in hair restoration. Skills and experience of the surgeon as well as the technician varies. A potential risk factor for the loss of density in the donor area is when the grafts are extracted too close to each other. Because smaller grafts are more difficult to extract than complete follicular units, to minimize the damage during the extraction and to achieve optimal results, only qualified and experienced technicians are extracting the grafts. This high level of training, with continuous evaluation regarding their skills and renewal of training contributes significantly to the high rate of extraction of usable grafts, the low complication rate and minimal scarring in the donor area. However, we still noticed some variability in the regrowth in the donor area, not only between patients but also between different consecutive procedures as well.

There could be several other reasons why regrowth in the donor area varies between patients:

1. Variability in the hair characteristics and donor skin:
   - **A.** Curly hair is more difficult to extract than strait hair.
   - **B.** Grey hair is more difficult to extract.
   - **C.** Blond hair has smaller follicle units than dark hair, and more difficult to extract
   - **D.** Donor area which has been used for previous operations with other techniques is more difficult to use compared to "virgin" donor areas.
2. 10-20% of the hair follicles in the donor area are in the telogen phase, which means that these follicles are very small. Unfortunately, you cannot distinguish these telogen hairs from anagen hairs with the eye. The chance of extracting too much tissue from telogen hair follicles to regenerate hairs after extraction is therefore higher than with anagen hair follicles. For example, by extracting 1400 grafts, which exists of 2 to 3 hair follicles per graft could lose 1400 grafts x 2-3 hairs x 10-20% = 350 to 700 hairs. This explains that after several treatments, some visible density loss will appear.
3. With any consecutive treatment, there are follicles where a part is already extracted. The morphology of these follicles is changed by fibrosis. Therefore, the use of the same follicles is more difficult. So, even with PL-FUT there is an end to the source.
4. The general health condition is also important. For example, wound healing is decreased in patients who smokes or who are diabetic.

In 39 cases of the 2306 evaluated PL-FUTs (1.7%) the regrowth in the recipient area was less than 75%, which we judged as a failure. The failure of growth of the implanted PL-FUTs could among other causes, be due to bad vascularization as well as necrosis when the grafts are implanted too close to each other. We were not able to count the exact number of grown hairs, because of the large number of implanted grafts.

Duing the 7 years evaluation period, adaptations were made in the technique. Introduction of an automated extraction device in 2006, the foot-controlled counters in 2007 as well as the introduction of new tweezers and anesthetic method with iontophoresis in 2009 and the increase in the amount of grafts, resulted in the high percentage of satisfied patients (2202 of 2306 patients; 95.5%) which is quite unique in the cosmetic practice.

Although there are no differences in most of the mentioned subjective as well as objective side effects, the decrease of swelling from 4 of the 26 cases in 2005 (15.4%) to 30 of the 694 PL-FUTs in 2011 (4.3%) could probably be due to a combination of the use of the anti-swelling medication prednisolone, the coldpack as well as a decrease of the necessary anesthetic in the recipient area. Although literature about the place of corticosteroids in cosmetic procedures are far from conclusive[13], up to now, we continue the use of corticosteroids post-treatment based on our experience.

No significant differences could be detected in the subjective density of the donor area, regrowth in the recipient area, and satisfaction between the PL-FUTs performed in 2005 and 2011. From the 2306 subjectively evaluated patients the density in 30 cases was decreased (1.3%). We regard this as a low percentage and in favor for the technique. However, a comparative study between PL-FUT and FUE is lacking.

As the PL-FUT needs small parts of the follicular unit, we developed special needles of 0.6mm in diameter for extraction.[7] The possibility to implant only parts of the follicular units leads to an excellent hairline and patient satisfaction. Because smaller 0.6mm grafts (0.28mm^2) can be placed more precise and with a higher density compared to the FUE technique which uses 0.8mm (0.50mm^2). The disadvantage of PL-FUT is that this procedure is a very labor intensive which limits the procedure to a maximum of 1600 grafts in one day. In contrast to other techniques, more consecutive treatments are necessary to obtain a full natural cosmetic result. We have not found large important series of conventional hair restoration techniques regarding side-effects, complications and patient outcome, and therefore comparison of our results with other techniques in literature is impossible. We can conclude PL-FUT represents a patient friendly and reliable method with satisfactory results to restore hairs with consistent results and preservation of the donor area. Therefore, we prefer PL-FUT above all other surgical techniques for hair restoration. It is an interesting idea to perform a clinical trial in which the comparison with PL-FUT and traditional FUE will be studied. Besides androgenic alopecia, PL-FUT has been used successfully on burn scars[14] as well also reconstruct eyebrows.[15] Preliminary result on frontal fibrosing alopecia (FFA) are promising, but more experience with the PL-FUT in (progressive) hair diseases is necessary to evaluate the limitations and further possibilities of this technique.

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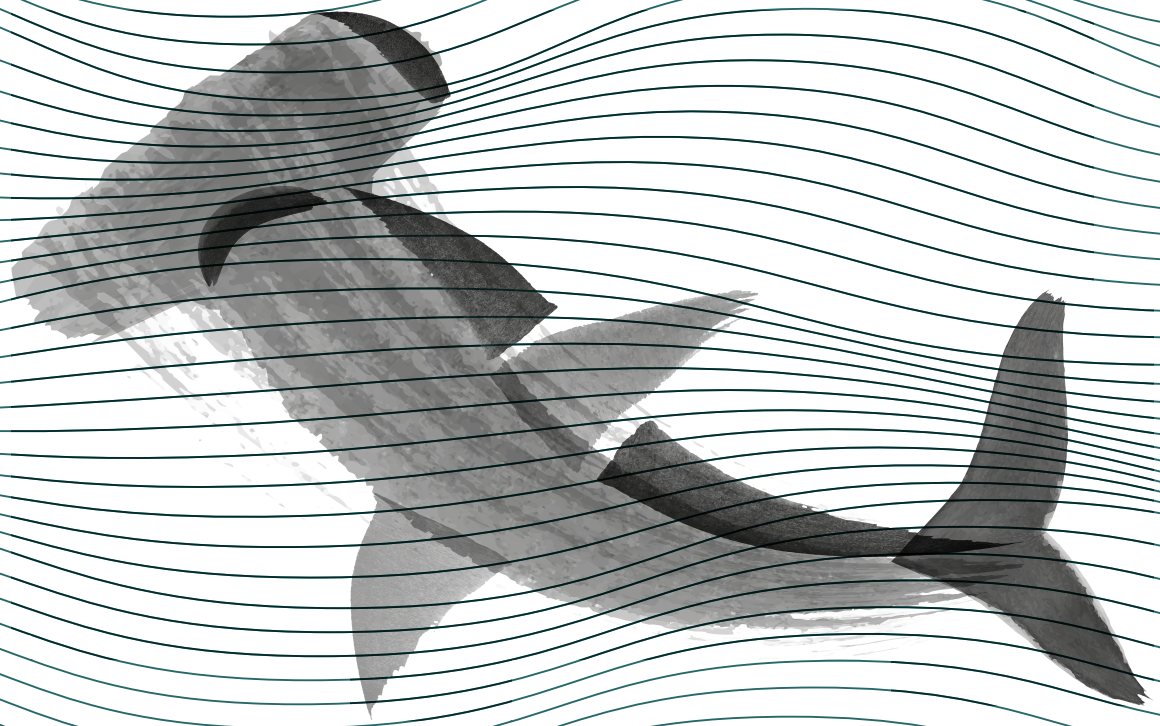

HAIR FOLLICULAR STEM CELLS

FOR EXPLORING THEIR POTENTIAL FUTURE APPLICATIONS IN REGENERATIVE MEDICINE

5 ISOLATION, EXPANSION AND NEURAL DIFFERENTIATION OF STEM CELLS FROM HUMAN PLUCKED HAIR: A FURTHER STEP TOWARDS AUTOLOGOUS NERVE RECOVERY.

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Gho CG, Schomann T, de Groot SC, Frijns JH, Rivolta MN, Neumann HAM, et al. Isolation, expansion and neural differentiation of stem cells from human plucked hair: a further step towards autologous nerve recovery. Cytotechnology. 2016;68(5):1849-58.

ABSTRACT

Stem cells from the adult hair follicle bulge can differentiate into neurons and glia, which is advantageous for the development of an autologous cell-based therapy for neurological diseases. Consequently, bulge stem cells from plucked hair may increase opportunities for personalized neuroregenerative therapy. Hairs were plucked from the scalps of healthy donors, and the bulges were cultured without prior tissue treatment. Shortly after outgrowth from the bulge, cellular protein expression was established immunohistochemically. The doubling time was calculated upon expansion, and the viability of expanded, cryopreserved cells was assessed after shear stress. The neuroglial differentiation potential was assessed from cryopreserved cells. Shortly after outgrowth, the cells were immunopositive for nestin, SLUG, AP-2α and SOX9, and negative for SOX10. Each bulge yielded approximately 1 x 10^4 cells after three passages. Doubling time was 3.3 (±1.5) days. Cellular viability did not differ significantly from control cells after shear stress. The cells expressed class III ß-tubulin (TUBB3) and synapsin-1 after 3 weeks of neuronal differentiation. Glial differentiation yielded KROX20- and MPZ-immunopositive cells after 2 weeks. We demonstrated that human hair follicle bulge-derived stem cells can be cultivated easily, expanded efficiently and kept frozen until needed. After cryopreservation, the cells were viable and displayed both neuronal and glial differentiation potential.

INTRODUCTION

During the last decade, the interest in autologous stem cells has increased considerably, especially regarding the development of individualized therapies.[1] However, the procurement of autologous somatic stem cells for human therapeutic purposes is still limited. In addition, somatic stem cell potency is restricted, and multipotent rather than pluripotent.[1] Reprogramming somatic cells into induced pluripotent stem cells by the forced expression of certain genes is being explored but is controversial, since they are often tumorigenic and may initialize a T cell-dependent immune response in syngeneic recipients.[2,3,4]

For these reasons, the use of other types of autologous somatic stem cells is currently under investigation, such as bone marrow stem cells in a curative treatment for ischemic heart patients and cerebral infarction,[5,6] already showing some promising results. One of the risks with this approach is that stem cells may follow their innate biological inclination irrespective of the tissue or organ into which they have been grafted. This was demonstrated by the finding that autologous bone marrow stem cells can produce extracellular matrix after engraftment into the brain.[7,8] An alternative strategy could involve the use of neural crest-derived stem cells (NCSCs). They are appropriate for autologous cell-based therapy in many diseases, as they can be derived from adult tissue and can give rise to many cell types from ectodermal and mesodermal lineages. NCSCs from adult tissue (aNCSCs) are nononcogenic and possess a broad regenerative potential.[9] In culture, aNCSCs retain their neural crest potential to differentiate into a variety of cells including adipocytes, chondrocytes, neurons, glia, osteocytes, and muscle cells.[9] Minimally invasive, easily accessible sources for NCSCs are the olfactory sheath, palate, dental pulp and the hair follicle bulge.[9] We consider the hair follicle to be the most easily accessible option.[10] It has been reported that hair follicle bulge-derived NCSCs (HFBSCs) from human adults still possess neural crest characteristics such as multipotency.[11,12,13,14,15] This multipotency is of particular use in the area of neuroregeneration, given that hair follicle stem cells can promote the functional recovery of injured peripheral and central nerves.[16,14,15,17] Hence, autologous HFBSCs are potentially suitable for therapeutic application in a broad range of neurological disorders such as ALS, Alzheimer's disease and stroke. They also may be used in cell-based therapies for sensory neurological diseases such as those for ocular or inner ear regeneration.[18,10] If HFBSCs could be harvested from plucked hairs, their practical utilization for autologous stem cell therapy would increase immensely. We therefore aimed to establish that:

1. Follicular stem cells, migrated out of the bulge from plucked hair follicles, are nestin-positive and possess a neural crest stem cell immunophenotype. Moreover, due to the cytotoxic nature of proteases, we intend to minimize the enzymatic treatments of tissue and cells.[19]
2. HFBSCs can be used for transplantation purposes, because they can be expanded easily and remain viable after cryopreservation and needle shear stress.
3. These stem cells are suitable as a source for future neural regenerative medicine in the patient, i.e. they can be stored frozen while keeping their neural and glial differentiation capacity.

MATERIALS AND METHODS

Specimen

Plucked hairs from healthy donors were obtained from the occipital area of the scalp. The hairs were removed with depilation forceps. All human material was handled according to the Dutch Medical Treatment Agreement Act (Dutch Civil Code, Book 7, Section 7.7.5, article 7:467; http://www.dutchcivillaw. com/legislation/dcctitle7777.htm). Intact hair follicles (HFs) in the anagen phase were selected under a dissection microscope, and placed in DMEM/Ham's F-12 1:1 (Biochrom AG, Berlin, Germany) containing 1% GlutaMAX (100x; Life Technologies, Carlsbad, CA, USA) and 1% Antibiotic Antimycotic Solution (100x; Sigma-Aldrich, St. Louis, MO, USA) (Fig. 1a). The HFs were processed the next morning.

Isolation and cultivation of HFBSCs

Isolation of HF stem cells was according to SieberBlum et al. (2004) with minor changes. Briefly, connective tissue (if present) was removed from the HF and the bulge-containing area was dissected out just below the sebaceous gland and well above the bulb (Fig. 1A). Then, a longitudinal section along the tissue of the bulge was made, to cause the tissue to unfold. During these procedures, care has to be taken to avoid dehydration of the HF. Before the start of the culture, tissue culture 12-well plates (TPP; Trasadingen, Switzerland) were coated with poly-D-lysine (PDL; Sigma-Aldrich) diluted in sterile demi water (1:10) at 37 °C and 5% CO_2 for 1 h. Then the PDL solution was removed and the wells air-dried under sterile conditions. Prior to usage, the PDL matrix was rehydrated with basic growth medium (BGM, 37 °C, 30 min). BGM consisted of DMEM/Ham's F-12 1:1, containing 1% GlutaMAX, 1% Antibiotic Antimycotic Solution, supplemented with 10% fetal bovine serum Gold (FBS; Life Technologies), 2% B-27 Supplement without vitamin A (50x; Life Technologies), 1% N-2 MAX Media Supplement (100x; R&D Systems, Minneapolis, MN, USA), recombinant human Fibroblast Growth Factor-basic (rhFGF-basic; 20 ng/ml; R&D Systems), and recombinant human Epidermal Growth Factor (rhEGF; 20 ng/ml; R&D Systems). After rehydration, the BGM was poured out of the wells, and one HF-bulge was placed in each well. The HFs were carefully pressed on the bottom of the well using a forceps. Subsequently, three incubation periods in a small drop of medium allowed the HF to attach to the matrix. Incubation was done at 37 °C and 5% CO_2 for 75 min. If necessary, some medium was added. Finally, 500 µl of freshly prepared BGM was added cautiously. The primary culture was established by the outgrowth of HF stem cells from the bulge, usually at 8–10 days after the start of the culturing. After 1 week of culturing, a complete medium change was performed, followed by replacement of half of the medium every other day. Three to four days after the start of outgrowth, the HF bulge was removed and some of the cultures were fixed with 1% formaldehyde in PBS (FA) for immunohistochemical analysis of neural crest markers.

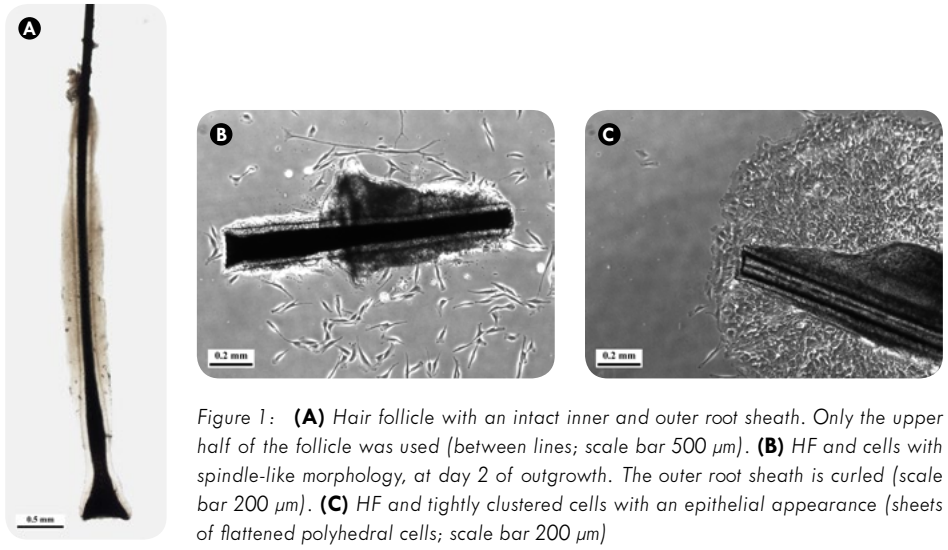

Figure 1: **(A)** *Hair follicle with an intact inner and outer root sheath. Only the upper half of the follicle was used (between lines; scale bar 500 µm).* **(B)** *HF and cells with spindle-like morphology, at day 2 of outgrowth. The outer root sheath is curled (scale bar 200 µm).* **(C)** *HF and tightly clustered cells with an epithelial appearance (sheets of flattened polyhedral cells; scale bar 200 µm)*

Expansion and cryopreservation

After removal of the bulge, cells were grown to 60–70% confluence and enzymatically detached using pre-warmed 0.05% trypsin–EDTA (Life Technologies) at 37 °C for precisely 2 min. Trypsinization was stopped by the addition of DMEM/HAM's F-12 1:1 supplemented with 10% FBS. The cells were centrifuged at 280 x g for 10 min, and the cell pellet was suspended in 1 ml BGM. After cell counting (Logos Biosystems, Anyang-City, Korea), the cells were seeded at expansion density (2.5 x 10^3 cells per cm^2) in a PDL-coated dish and allowed to expand until 60–70% confluence. In general, cells were passaged three to four times. Each period of time prior to passaging was about 1 week. Doubling times were calculated at passages 2 and 3, using the site: Roth V. 2006 Doubling Time Computing, Available from: http://www.doubling-time.com/compute.php.[20]

In addition, a portion of the cells was frozen at -80 °C at a concentration of 1 x 10^6 cells/ml in 90% FBS with 10% dimethyl sulfoxide (Sigma-Aldrich). After storage and thawing, the cells were suspended in 5 ml BGM, centrifuged, collected, suspended in BGM, carefully triturated, seeded at expansion density, and cultured at 37 °C and 5% CO_2.

Simulation of the transplantation procedure: ejection of cells

After cryopreservation, cells were cultured at 37 °C and 5% CO_2. After 1 week cells were enzymatically detached and centrifuged at 280 x g for 10 min. They were suspended at a density of ~4.0 x 10^6 cells/ml in BGM medium and carefully triturated. Subsequently, 10 µl of the cell suspension was loaded into a 100 µl syringe with a 30 gauge needle and injected into a 1 ml Eppendorf tube using a programmable syringe pump (Prosense, Oosterhout, The Netherlands); settings: diameter 4.699 mm—rate 0.5 ml/min. Both cultured and cryopreserved cells were subjected to shear stress. Viability was assessed using the trypan blue test. Trypan blue staining is based on the principle that live cells possess intact cell membranes that exclude the dye, whereas dead cells do not.[21] A 1:1 dilution of cell suspension and 0.4% trypan blue was incubated for 2 min at room temperature. Next, the stained cells were counted using a Neubauer haemocytometer chamber and calculated using the following formula: vital cell rate (%) = number of vital cells/(number of vital cells + number of dead cells) x 100%. Cells that had been cultured but not injected served as controls.

Statistical analysis

The paired, two-tailed Student's t test was used to estimate the difference between the control and injected cells. The unpaired, two-tailed Student's t test was used to estimate the difference between control cells and both cryopreserved and injected cryopreserved cells.

Neural differentiation of HFBSCs

Following outgrowth, expansion, and cryopreservation of HFBSCs, 2.5 x 10^5 cells in 500 µl of BGM were seeded per well of a 12-well plate. The cells were seeded via the side into PDL-coated wells containing PDL-coated cover glasses (Thermo Scientific, Waltham, MA, USA). It was essential in all the described procedures to prevent the cover glass sticking to the bottom of the well. Prior to PDL coating, the cover glasses were etched in 85% phosphoric acid (Merck Millipore, Darmstadt, Germany) for 12 h.[22] Subsequently, acid-treated cover glasses were rinsed extensively in ultrapure water and subjected to a graded series of 70, 90, and 96% ethanol. Cover glasses were stored in 96% ethanol. The 12-well plates and etched cover glasses were coated separately with PDL as described previously. After seeding, the cells were cultured at 37 °C and 5% CO_2, while their settlement underneath the cover glass was observed daily. When an appropriate density was achieved, i.e., 5 to 10 cells in one field of view (FOV, 10x magnification, an area of ~3.5 mm2), differentiation was induced by removal of 250 µl medium and replacement with 300 µl cAMP-containing induction medium (IM).[23] IM consisted of DMEM/Ham's F-12 1:1 supplemented with 1.5 mM cAMP (Sigma-Aldrich), 1% glutamax (Life Technologies), 10 ng/ml NGF, 10 ng/ml GDNF, 10 ng/ml BDNF (all from R&D Systems) and 2% B27 + VitA (Life Technologies). If the appropriate density was not achieved, half of the medium was replaced with fresh BGM every other day. After IM was added, the cultures were allowed to differentiate for at least 60 h without disturbance due to opening of the incubator or observation of the cells. Subsequently, 250 µl medium was removed and again substituted with 300 µl IM. Thereafter, the medium was replenished with IM every other day. Cultures were observed for morphological changes on a daily basis. If no neuronal morphologies appeared after 7 days of differentiation, the culture underwent another period without disturbance in IM, and the above-mentioned differentiation procedure was followed again. After differentiation for 7–14 days, the cover glass was carefully removed, because cells were not only attached to the bottom of the well but sometimes also to the underside of the cover glass. The cells on the bottom of the well were fixed in 1% FA for 15 min and processed for immunohistochemistry. Fixed cells were stored at 4 °C for a maximum period of 2 weeks.

Glial differentiation of HFBSCs

After expansion, a volume of 500 µl of BGM (without FBS) containing 1 x 10^5 cells was pipetted into each well of a 12-well plate. The cells were seeded via the side into uncoated wells which contained PDL-coated cover glasses. During culture, the cover glass should not stick to the bottom of the well. On the next day, half of the medium was replaced with serum-free BGM .[24] Cells were observed every other day to follow settlement and changes in morphology. The medium was exchanged with serum-free medium every other day until an average density of 10–20 cells per FOV underneath the cover glass was reached. Then half of the medium was replaced by IM. The cells underneath the cover glass usually adopt glial-like morphologies after 3–4 days of induction. After induction, the medium was exchanged once a week with IM. Cultures were maintained until networks of cells were observed. Because the networks were attached to the underside of the cover glass, the cover glasses were removed and placed upside down in another well of a 12-well dish. The cells were fixed in 1% FA for 15 min.

Immunohistochemistry

Prior to immunohistochemistry, the cells were washed with 0.05% Tween-20 in PBS for 5 min and permeabilized in 0.1% Triton X-100 in PBS for 10 min. Cells were treated with blocking solution consisting of 5% non-immune serum in 0.05% Tween-20 in PBS for 30 min. Afterwards, the cells were incubated with the primary antibodies in blocking solution at 4 °C overnight.[25] The primary antibodies used were: anti-Nestin (1:500, Biosensis, Thebarton, South Australia), anti-SLUG (1:125, Abcam (Cambridge, U.K.) ab 27568), anti-AP-2α (1:100, Santa Cruz Biotechnology (Santa Cruz, CA, USA) sc-53164), anti-SOX9 (1:500, Millipore (Billerica, MA, USA) AB5535), anti-SOX10 (1:200, Santa Cruz sc-17342), anti-β-III-tubulin (1:200, Abcam ab18207), anti-synapsin-1 (1:200, Abcam ab8), anti-myelin protein zero (MPZ; 1:200, Neuromics (Minneapolis, MN, USA) Ch23009), and antiKrox20 (1:100, Covance, New York, NY, USA). The secondary fluorochrome-conjugated antibodies were diluted 1:500 in blocking buffer, and the cells were incubated at room temperature for 1 h. The secondary antibodies were conjugated with either Alexa Fluor 488 or Alexa Fluor 555 (Life Technologies). Nuclear counterstaining was performed with 1:1000 DAPI (Life Technologies, D3571) in PBS. The cells were covered with Vectashield (Vector Laboratories, Burlingame, CA, USA). Omission of the primary antibody served as a control for false cross-reactivity of the secondary antibody. Pertinent positive cell or tissue controls were used: RT4-D6P2T, a rat Schwann cell line (ATCC, Manassas, VA, USA) for anti-nestin, -SOX9, -Krox20, and -MPZ, the Melan-Ink4a cell line (Wellcome Trust Functional Genomics Cell Bank, London, UK) for anti-SOX10 and -β-III-tubulin,[28] the SKBR3-breast cancer cell line for anti-SLUG and Ap-2α, and mouse brain for anti-synapsin-1.

Fluorescence imaging was performed using fluorescence microscopy (Olympus IX70) in combination with the LAS AF microscope software (Version 1.9.0 build 1633, Leica Microsystems). The data were corrected for background staining and normalized using the quantification method of the software. Only those immunostainings showing a peak maximum at emission of at least two times higher than the background were considered to be significantly positive. Pictures were processed using Adobe Photoshop CS6 Extended (Version: 13.0 x64, Adobe Systems Incorporated, San Jose, CA, USA).

RESULTS

Isolation and cultivation of HFBSCs

In our experience, more than 60% of the bulges remained attached and produced cellular outgrowth (Table 1). However, outgrowth did not always yield the desired phenotype of HFBSCs with spindle-like morphology (Fig. 1B); tightly clustered cells with an epithelial appearance were also seen (sheets of flattened polyhedral cells, Fig. 1C). Both cell phenotypes emigrated from bulge explants at 8–10 days of culture. Based on morphology and immunohistochemistry (results not shown), the flattened cells were identified as keratinocytes. Therefore, cultures containing those cells were discarded (Table 1).

Cultures	HFs planted (n)	Cultures with outgrowth (%)	Cultures with polyhedral cell outgrowth (%)	Cultures with spindle-like cell outgrowth (%)
905E1P0	12	58	33	57
906E1P0	12	58	57	43
916E1P0	12	67	37	63
932E1P0	12	67	100	0
934E1P0	30	67	75	25
Average	–	63	Discarded	34

Table 1: Outgrowth, adhesion and morphology of cells from the HF bulge explant

In vitro NCSC-characteristic protein expression profile

The majority of HFBSCs express the neural crest cell markers SOX9, SLUG, and AP-2α as determined by indirect immunohistochemistry.[26] SOX10 expression was below the level of detection. The neural progenitor cell marker nestin was present in all cells (Fig. 2A–E).

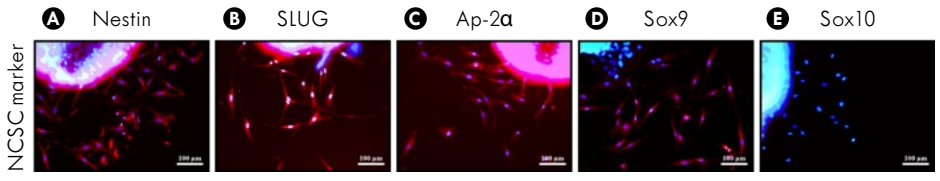

Figure 2: HFBSCs at day 2 of outgrowth. The NCSC markers nestin (red), SLUG (red), AP-2α (red) and SOX9 (red) are positive. SOX10 (red) is negative. Nuclei are stained with DAPI (blue; scale bar 100 µm)

Expansion and cryopreservation

The selected primary (P0) cultures reached 60–70% confluence after 15–19 days. The P1 cultures reached 80% confluence after 1 week and those from P2, 1 week later. The total yield of cells per HF was approximately 1 x 10^4. The mean doubling time, calculated from the P2 and P3 cultures, was 3.3 (±1.5) days (Table 2).

Cultures	Cells at start of culture (n)	Cells at end of culture (n)	Doubling time (days)
905P1 to P2	199,000	1,872,000	2.8
916P1 to P2	328,000	2,128,000	5.6
934P1 to P2	60,000	1,000,000	2.7
1011P2 to P3	350,000	3,800,000	2.9
1012P1 to P3	350,000	4,400,000	2.7
Avg ± SD			3.3 ± 1.5

Table 2: Doubling times of HFBSCs after passaging

Simulation of the transplantation procedure: ejection of cells

Shear stress caused by injection through a syringe needle did not change the viability of the cells significantly, whether the cells were freshly cultured or frozen ($p = 0.2401$ and $p = 0.6306$, respectively). However, a small but significant difference in viability was found between cells which were only cultured and those which were cryopreserved ($p = 0.009$). Nevertheless, after cryopreservation, 82.2% ± 2.33% of the cells were still viable. These results show that HFBSCs can be expanded and kept frozen until needed.

Neural differentiation

For neural differentiation, we used the principle of the 'sandwich method'. The rationale for this method is, that neuronal survival is improved if the cells are grown on a substrate-coated surface and covered by a cover glass.[27] In general, a density of about 8 cells per FOV (~3.5 mm^2) underneath the cover glass gave the highest number of surviving neuron-like cells during neuronal induction. A considerable number of cells on top of the cover glass could not endure neuronal induction and died, resulting in cellular debris. Too much of this debris appeared to be destructive to neurons, because it covered the developing neuron-like cells and their projections, vacuoles were formed and the neurons subsequently deteriorated. Under the condition that cells were not covered by cellular debris, different neuron-like cells with elongated, branched projections developed over time. These projections could grow to a length ranging between 100 and 500 µm (Fig. 3A). Neuron-like cells were found mainly on the bottom of the well.

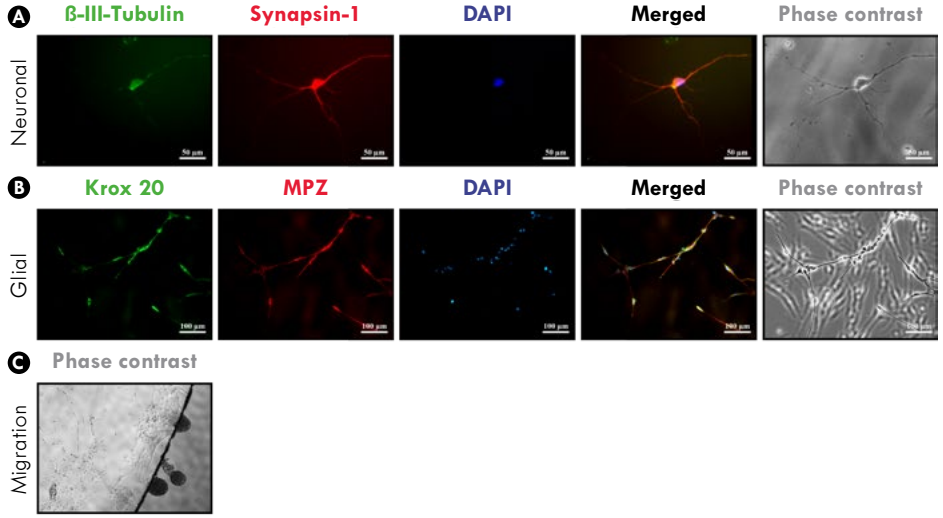

Figure 3: ***(A)*** *Representative HFBSCs 14 days after neuronal induction. Cells at the bottom of the well stained positive for bIII-Tubulin (green) as well as synapsin-1 (red). The cell nucleus is stained with DAPI (blue). Merged image reveals overlay of bIII-Tubulin (green) as well as synapsin-1 (red) localization. The phase contrast image shows differences in long dendritic projections and thicker axon (scale bar 50 µm).* ***(B)*** *HFBSCs 15 days after glial induction. Glial cells were at the underside of the cover glass, therefore cells on the top of the glass are also faintly visible, but out of focus. Cells stained positive for the glial markers Krox20 (green) and MPZ (red). Nuclei are stained with DAPI (blue). Cells with a yellowcolor in the merged image co-express MPZ (red) and Krox20 (green). The phase contrast image depicts spindle-shaped morphologies of the glial cells (scale bar 100 µm).* ***(C)*** *Migration of glial cells underneath the cover glass*

Glial differentiation

After seeding many cells attached within a few minutes to the PDL matrix, but a considerable number of HFBSCs remained motile. A considerable number of these cells migrated underneath the cover glass, sometimes in globular cell aggregates.[24] These cells were mainly bipolar, with a distinct bulbous soma and small projections. After 2 or 3 days, most of these cells showed premature glial-like morphologies. A few days later, their soma became spherical and the first projections appeared, all very different in length. These glia-like cells quickly formed spacious, structured networks. With further culturing, the cells and networks did not alter noticeably.

Immunohistochemistry

In general, many cells were β-III-tubulin-positive early during neural induction, and synapsin-1-positivity was detectable a few days later (Fig. 3B–D). Glial induction yielded cells which were β-III-tubulinnegative, but positive for MPZ and Krox20 (Fig. 3B–D).

DISCUSSION

We isolated, expanded and cryopreserved nestinpositive stem cells, derived from the bulge area from plucked human hairs. We showed that these stem cells, which are also positive for the neural crest markers SOX9, SLUG and AP-2α, are suitable for transplantation purposes because they easily survive cryopreservation and needle shear stress, while conserving their neuronal differentiation capacities.

The technique used to harvest the HF is virtually painless and allows the collection of hundreds of HF per patient. The culture technique to expand the cells is simple and straightforward and usually yields 1 x 10^4 cells per HF. Only three to four passages are required for this high yield, so cellular senescence is not an issue. Moreover, cellular damage by frequent protease treatments is restricted to a minimum. We do not consider the series of conditions mentioned in this report as imperative protocols, prerequisite to stimulating these cells towards differentiation into neurons and glia cells. There are some critical points in the procedures, however, such as the selection of intact hair follicles, rejection of cultures with undesired cell outgrowth, and the sandwich differentiation culture method. Rigorous rejection of cultures based on critical observations and knowledge of contaminating cell types allowed us to perform a "selection at the gate" of the desired cell type. Obviously, the group of cells which are most prominently present in the hair follicle are keratinocytes. These cultures were immediately discarded. Other cells present in the hair follicle are fibroblasts, which possess in the migrating phase–like many other cells–a spindle-like morphology. However, cultures containing fibroblasts will soon be overgrown by this contaminating cell type, for the doubling time of human dermal fibroblasts is in general 24 h, while the hair follicle bulge stem cells have a doubling time of an average of 3.3 days (see also "Results", section Expansion and cryopreservation). Fibroblasts will thus overgrow the other cells and form arrays of cells oriented in a curvilinear pattern. These cultures were also discarded. Other cells, which have been reported to be present in the hair follicle are melanocytes, cells from the peripheral nerve ends and some muscle cells.[28]

Melanocytes, which in the hair follicle have a similar morphology as the bulge cells (bipolar), do not grow under the culture conditions reported here: they need medium with a relatively low pH, generated by 10% CO_2. We did not consider Schwann cells from the peripheral nerve endings as contaminating, for they are also neural crest-derived. Muscle cells are sometimes present in mouse vibrissae cultures, but we have never demonstrated them in human hair follicle cultures (using αSMA staining).

Using the sandwich differentiation method, it was possible to vary the culture circumstances in such a way that a different neural cell phenotype was achieved. It is known that a combination of serum deprivation, cell density and substrate can direct neural stem cells to develop towards a neuronal or glial phenotype.[29] In the neuronal differentiation protocol, the surface of the cover glass was soon covered with many cells, due to the relatively high seeding density and proliferative stimulus of FBS. This apparently prevented the attachment of many cells. Thus, different cell types remained floating and finally found a habitat underneath the cover glass. Some of these cells showed a fibroblast-like morphology, while others were neuronlike cells with a small, shining soma and two or more thin projections. In time, the fibroblast-like cells disappeared; they apparently did not survive the differentiation medium. However, there was obviously contact between the small neuron-like cells and the fibroblasts, suggesting paracrine interactions which may stimulate neural differentiation.[30] We assume that, because of the decrease in cell density, neuron-like cells develop long and complex branches in order to seek contact with other cells. It is conceivable that the micro-environment underneath the cover glass, such as a low oxygen pressure and the build-up of autocrine factors, might facilitate neuroglial differentiation and survival of HFBSCs.[27] Interestingly, in both protocols, cells with a shining soma and thin projections preferred migrating underneath the cover glass while other cells apparently preferred to settle on top. This cell type-specific affinity to seek a place in the narrow space between the cover glass and the bottom of the well, or on top of the cover glass, also occurred when HFBSCs were seeded underneath the cover glass (results not shown).

We are convinced that the repertoire of nestinpositive stem cells from the human HF bulge will be of benefit for many different autologous cell-based therapies. Still, there seems to be a difference of opinion about the precise localization of these cells. Clewes et al.[31] described nestin-positive cells migrating from the HF bulge in culture, whereas Amoh et al.[14,15] found nestin-positive cells immediately below the sebaceous glands just above the bulge area, i.e., in the isthmus region. In our experience, it is nearly impossible to distinguish the bulge area from the isthmus region in human HFs (Fig. 1A). We therefore assume that the nestin-positive cells in our cultures and those described by Clewes et al.[31] do not differ from those described by Amoh et al.[14,15] as was also previously suggested by Djian-Zaouche et al.[32] Remarkable positive results of neural regeneration and functional improvement have been obtained in animal models of brain, spinal cord, and nerve injury therapy using stem cells derived from hair follicles,[14,15,16,17] giving hope for patients suffering from neurodegenerative diseases. Another point of interest, however, is the predisposition of these HFBSCs to repair cranial bone, nerves and tissue of the head, e.g. in patients with severely burnt faces. These patients often have enough remaining hair to perform hair transplantations and to harvest stem cells, which may open possibilities for complete autologous facial repair. This holds true for autologous stem cell therapy in general, as experience with the culturing of stem cells in autologous serum is increasing.[33]

CONCLUSION

We have demonstrated that stem cells from HFs, plucked from human scalps, display a NCSC immune profile and can be cultivated, expanded, and kept frozen until needed. We also showed that differentiation of these stem cells into neurons and glial cells is feasible. The technique used to harvest the HFs is almost painless and allows the collection of hundreds of HFs per patient, which is highly advantageous, making these cells very attractive for autologous transplantation and treatment models for a variety of disorders.

Acknowledgments

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GENERAL DISCUSSION

6 GENERAL DISCUSSION

Adapted from Gho, C. G. and H. A. M. Neumann (2015). "Advances in hair transplantation: longitudinal partial follicular unit transplantation." Curr Probl Dermatol 47: 150-157.

GENERAL DISCUSSION

In this thesis it is reported that human hair follicles contain distinct populations of stem cells, each of different potency: pluripotent, multipotent, oligopotent and unipotent stem cells. When in vivo transplanted as partial follicular units, these stem cells are able to generate new, fully differentiated, hair. The in vitro experiments show clearly the location of the stem cell populations and their capacity to differentiate into different dermal lineages. Based on this work we hypothesize that, providing a pertinent matrix that it is not necessary to transplant the whole hair follicle to regenerate new hairs but that a part of the follicular stem cells from the bulge compartment and from the bulb compartment will be sufficient to generate new hair.

The localization of the follicular stem cells in plucked and hair follicular cell culture.
The search was based on the follicular stem cell population in plucked hair and their cell cultures to investigate stem cell characteristics proposed in the recent literature[1], i.e. the expression of CK19 and Bcl-2 in combination with the absence (or low levels) of Bax expression[2] and an extremely low proliferation frequency.[3] In 1996, Michel et al[4] suggested that CK19-positive cells in hair follicles represented stem cells.

Our studies showed that Ki-67 and Bax positive cells were present in the dermal papilla. Bcl-2 positive cells were absent in this area and this finding indicated that there was no conformity with the definition of follicular stem cells. It supports the findings of others that follicular stem cells were not present in the dermal papilla.[56] However, this contradiction indicates that hair growth requires the co-operation of various stem cell regions, which are not present in the papilla.

Although plucked hair seems to contain all of the epithelial structures, which are present in the full hair follicle derived from skin biopsies, the transplantation of plucked hair does not result in normal hair growth in contrast to hair-containing punch grafts.[7]

Unsuccessful regeneration after implantation of plucked hairs may also be caused by the inflammatory response of the receptor area to the plucked hairs, which may result in the destruction of the follicular stem cells. This may be unlikely because micro-grafts, which are only just a little bigger can be used successfully as grafts. The connective tissue surrounding these epithelial hair follicle structures in micro- grafts may be crucial or as protection against inflammation or to induce cytokines to activate the development of a hair follicle and thus a successful transplantation. Therefore, the importance of the connective tissue surrounding the hair follicle cannot be ignored.[8,7] Future research with extracellular matrix components as well as stem cell enhancing factors will be performed to examine the possibility of using follicular stem cells derived from plucked hairs to regenerate hairs because plucked hairs are an excellent source of cells for culturing.

The viability of the hair transplantation grafts and follicular units.
There is an inversely proportional correlation between the cosmetic result of hair transplantation and the size of the graft. When using large grafts, the amount of viable tissue seems to be sufficient to regenerate new hair growth in conventional hair transplantation techniques.[9] However, smaller grafts are much more vulnerable. Grafts harvested from the donor area are devoid of blood supply and thus also from oxygen and nutrients. This lack of oxygen, which was also encountered in cardiology where the physiology of ischemic- and reperfusion injury was extensively studied.[10] Bis(maltolato)oxovanadium (BMOV) has been shown to inhibit tyrosine phosphatase, which be protective against apoptosis, even when given late after the onset of lack of oxygen, but the mechanism of protection is unknown.[10]

The hair transplantation grafts stored in the preservation solution containing BMOV had a significantly better viability than those with the two commercially available preservation saline solution and Ringer's lactate. We noted that a considerable amount of tissue remained viable in saline solution. Since it was proven that follicular stem cells were located at different areas in the hair follicle and that the preservation solution was essential for hair transplantations with smaller grafts, we believed that smaller grafts, which contained sufficient follicular stem cell, but not necessarily whole hair follicles, could initiate complete re-growth of hairs. Additional studies with (improved) preservation solutions with compounds such as extracellular matrix (ECM) components must be performed to evaluate the minimal quantity of tissue necessary to regenerate hair growth or even several hairs.

The hair growth potential of follicular stem cells, partial follicular units and their clinical applications in hair restoration.
Our studies showed that the hair stem cells occur in multiple areas in a hair follicle[11] and preservation solution is essential for hair transplantations with smaller grafts. It was shown that partial longitudinal follicle units, which contained sufficient follicular stem cell could initiate complete re-growth of hairs. On the basis of this, the partial longitudinal follicle unit transplantation (PL-FUT) was developed.[12] This technique is also called hair stem cell transplantation because only a portion of the total amount of donor hair stem cells is transplanted.

Longitudinal Partial Follicular Unit Transplantation (PL-FUT) consists of the following steps:

A. **Preparation of the donor site.** The donor site, mostly the occipital side of the scalp was shaved and disinfected with Chlorohexidine 2% lotion and anesthetized with Lidocaine 2% with adrenaline (AstraZeneca).

B. **Extraction of the partial longitudinal follicular units (grafts)**.The grafts are harvested with hollow triple-waved tipped, partially blunt needles with an inner diameter of 0.6mm (Hair Science Institute®, Amsterdam, the Netherlands) under 2x magnification. Using this needle and the coarse, dead hairs as a guide enabled the extraction of partially longitudinal follicular unit, even when the follicular unit was not in a perfect triangular configuration. The needle was placed around the visible hairs and then twisted in the

same direction until the grafts were detached from the dermis. The grafts were extracted with micro-surgical forceps. The aim of the extraction was to remove only a part of the follicle unit containing follicle and connective tissue from several hair follicles and leave sufficient follicle unit tissue behind to re-generate hairs. After the extraction, Fucidin Cream® (Leo Pharma, Breda, the Netherlands) was applied on the donor area. After extraction, the partial longitudinal follicular units were stored in the preservative medium, containing the following ingredients; Sodium Chloride, Potassium Chloride, Magnesium Sulphate, Sodium Phosphate, Calcium Chloride, Glucose, Sodium Bicarbonate, Sodium Lactate, Sodium Pyruvate, Human Serum Albumin, Insulin, Bis(maltolato) oxovanadium (BMOV) and α-Tocopherol (vitamin E) (Hair Science Institute®, Amsterdam, the Netherlands) for two hours until implantation.

C. **Preparation of the recipient area.** The recipient area was disinfected with Chlorohexidine 2% lotion and anesthetized with Prilocaine hydrochloride 30 mg/ml (3%) and Felypressine 0.54 µg/ml (Citanest) (AstraZeneca) or Lidocaine 2% with adrenaline (AstraZeneca). Miniscule holes were made with a hollow needle with an inner diameter of 0.5 mm (Hair Science Institute®, Amsterdam, the Netherlands) and skin was removed or the grafts were implanted directly after punching the holes with a 21G hypodermal needle.

D. **Implantation of the grafts.** After the preparation of the recipient area, the grafts were implanted with micro-surgical forceps.

Differences with traditional hair transplantation techniques.

Traditional hair transplantation techniques require strip removal at a depth of 1–1.5 cm to obtain hair follicles. FUE is another technique in which the whole follicular unit is extracted without leaving sufficient tissue behind to regenerate new hair or follicular units, resulting in linear or circular scars in the donor area (Fig. 1A, B).

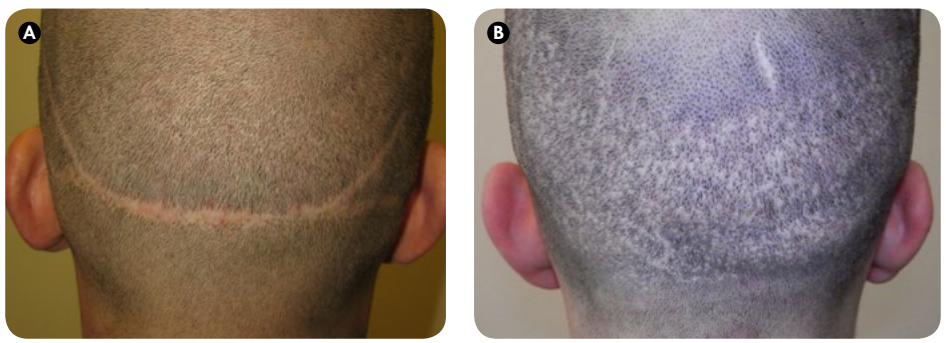


Figure 1: **(A)** *A male patient with a linear scar due to a previous Hair Transplantation with the "Strip method".* **(B)** *A male patient with circular scars due to a previous Hair Transplantation with the "FUE method ".*

In contrast to these techniques with PL-FUT successful transplantation was feasible using longitudinal partial follicular units with a diameter of 0.5 to 0.6 mm and 5 to 6 mm in length. Due to minimal skin and tissue removal, there was minimal to no scarring, pain or other postsurgical trauma such as nerve and vascular damage. There was also no possibility that the scars would "stretch out" over time (Figures 2 & 3).

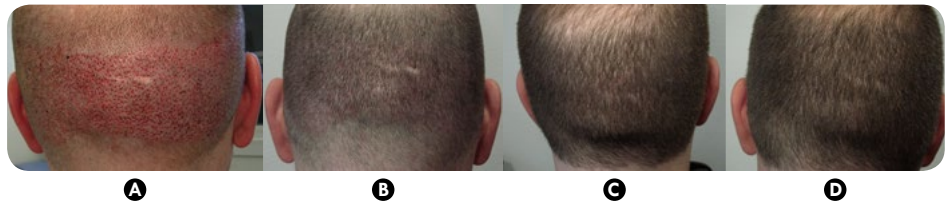


Figure 2: Donor area for the harvesting of hair follicles for partial longitudinal follicle unit transplantation as a treatment for androgenetic alopecia **(A)** *donor area immediately after extraction;* **(B)** *1 week after extraction;* **(C)** *3 months after extraction and* **(D)** *1 year after extraction.*

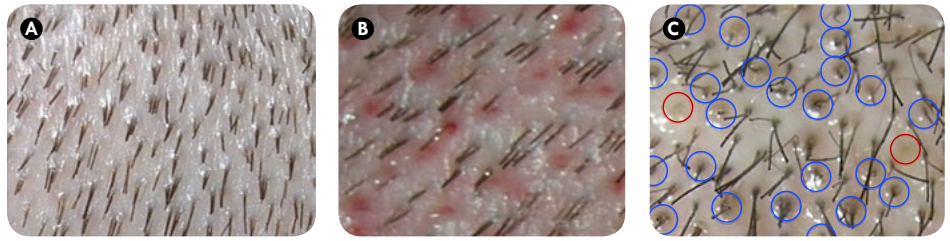


Figure 3: Close-ups of a donor area before the harvesting of hair follicles for partial longitudinal follicle unit transplantation as a treatment for androgenetic alopecia **(A)** *the area before extraction;* **(B)** *immediately after extraction;* **(C)** *1 week after the extraction. In the last picture, the dark spots are the places where the 'grafts' have been removed. In these dark spots, we see little hairs growing (blue circles); that is the re-growth. Some dark spots do not contain small hairs (red circles). Here the re-growth takes longer, probably because more tissue was removed.*

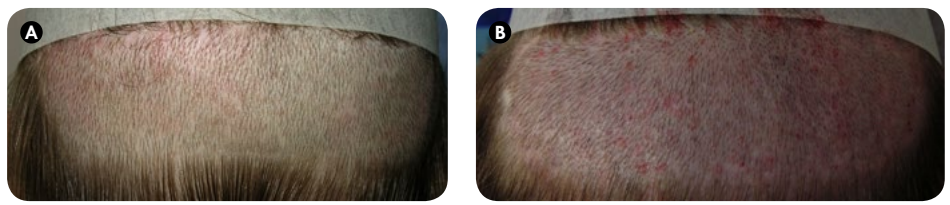


Figure 4: **(A)** *The donor area of a patient before, and* **(B)** *three years after 3 PL-FUT treatments and in total 3858 grafts were extracted. After evaluation of the donor area almost all the hair follicles in the donor site re-produce hairs after three years.*

There remained enough hair stem cells at the donor location to produce the same number of hairs with the same diameter and characteristics because the entire hair follicle unit was not removed with PL-FUT (Fig. 5 & 6). These follicular units in the donor area could be used again in consecutive treatments. Thus, 2 hair follicles were made from 1. These are the essential differences between PL-FUT and all other hair transplantation methods.[12]

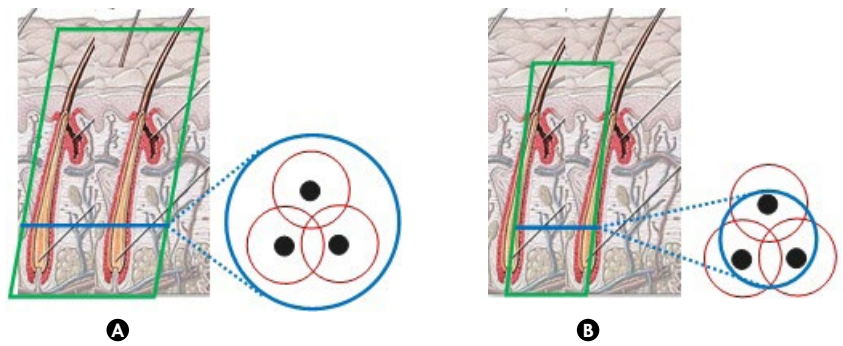


Figure 5: The difference between **(A)** *hair follicle unit extraction and* **(B)** *partial longitudinal follicle unit transplantation as donor material for the treatment of androgenetic alopecia: the green line indicates the border of the graft and near the blue line to the right is a cross section of the follicle unit cut out. With follicle unit extractions, entire follicles are transplanted (diameter 0.7-1.2 mm) and with partial longitudinal follicle unit transplantation parts of the follicles (diameter: 0.5 to 0.6 mm).*

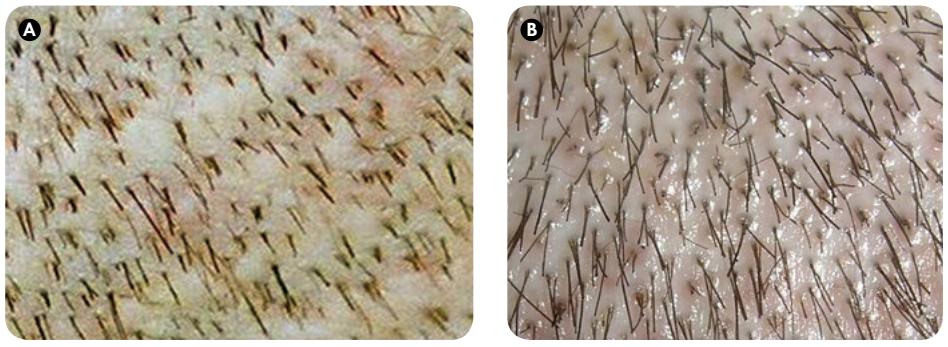


Figure 6: Donor location after harvesting hair follicles for transplantation as a treatment for androgenetic alopecia **(A)** *There is no complete regrowth after follicle unit extraction,* **(B)** *The hair growth has fully returned after partial longitudinal follicle unit transplantation.*

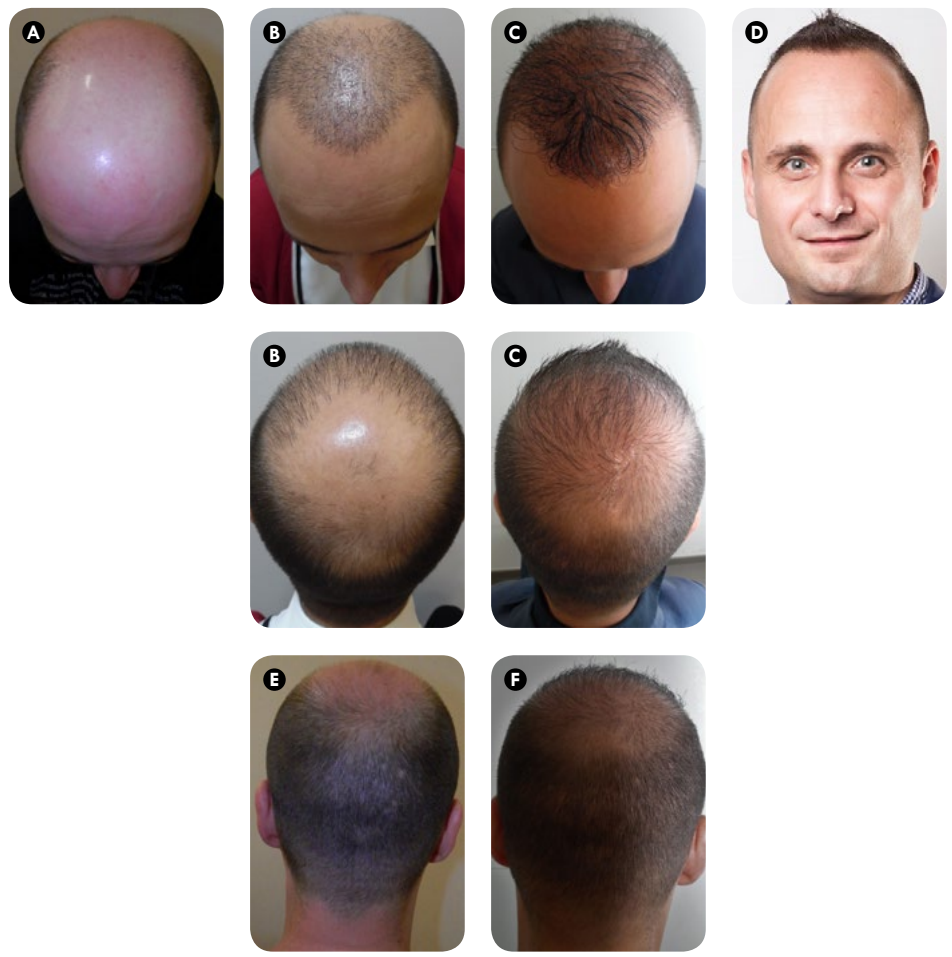


Figure 7: shows a male patient with Androgenetic Alopecia (27 years of age at his first treatment) who had two consecutive treatments with a total of 4868 grafts.

(A) Recipient area of a patient before treatment.

(B) Frontocranial and crown area after the first treatment of 2325 grafts.

(C) Frontocranial and crown area after the second treatment of 2543 grafts.

(D) Final result of frontocranial area after two consecutive treatments with a total of 4868 grafts. Although the maximum density had not been achieved, he was satisfied on the patient friendliness of the technique as well as the end result.

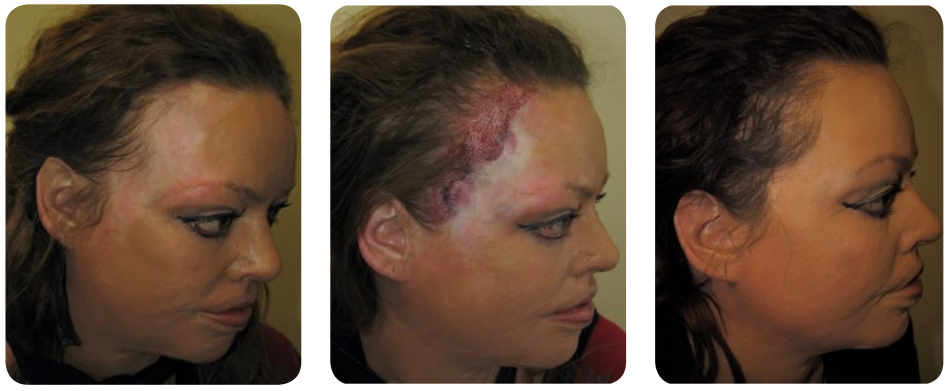
(E) Donor area of a patient before and 2 PL-FUT treatments and extraction of 4868 grafts.

(F) Donor area of a patient after 2 PL-FUT treatments and extraction of 4868 grafts. After evaluation of the donor area a major part of the hair follicles in the donor site re-produce hairs after extraction.

Normally, in traditional hair transplantation, the majority of implanted hairs would fall out quickly after implantation and re-growth would occur within three to six months. Since we did not implant complete follicular units, re-growth started after five to eight months after implantation, but sometimes took more than 12 months. This phenomenon was reported in other hair transplantation studies with dissected hair follicles.[13] Another difference of PL-FUT as compared with the traditional hair transplantation techniques, was that PL-FUT was a very labor intensive procedure and it took a whole day to transplant adequate number (up to 2,500) of grafts. These differences made it possible to use PL-FUT for androgenetic alopecia (AGA), facial and scalp burns or individuals with progressive scarring alopecia like FFA as well as for the restoration of eyebrows.

Partial longitudinal follicular unit transplantation (PL-FUT) in Androgenetic Alopecia. PL-FUT had great advantages such as the preservation of donor hair follicles, no scars and no stitches, almost no pain (both during as well as after the treatment) and better and faster healing of wounds over the traditional hair transplantation techniques.[14]

Partial longitudinal follicular unit transplantation (PL-FUT) in burn patients. (Fig. 8)
Although hair transplantation was the best option for facial burn scars, it was really a challenge for the surgeon because scar tissue behaved differently from the normal skin. Since PL-FUT represented the first reliable method to multiply hair follicles in vivo while preserving the donor area, PL-FUT was especially suitable for individuals with very limited donor area, for example burn victims.[15]



Before PL-FUT *Directly after PL-FUT* *Two years after PL-FUT*

Figure 8: A female patient with burn scars (42 years of age at her first treatment) who had a total of 1,021 grafts transplanted in the temporal area of the scalp, the right eyebrow and right sideburn spread over 2 consecutive treatments during a period of 2 years. The temporal area, the right sideburn and right eyebrow were fully restored and the maximum density (for burns) was achieved. She was satisfied on the patient friendliness of the technique as well as the end result.

Partial longitudinal follicular unit transplantation (PL-FUT) in Frontal Fibrosing Alopecia (FFA). (Figure 9)
Since FFA is an inflammatory skin disease of an unknown origin one had to realize that transplantation was only an option in the case the disease had lost all its activity.[16] To avoid a possible Köbner phenomenon it was advisable to prescribe unilateral Cyclosporine A be taken one week before as well as 1 month after PL-FUT. Since FFA is a slowly progressing disease, it was possible that more than one session of hair transplantation might be needed. Hair transplantations with the traditional methods that deplete the donor area will always limit the availability of donor hair follicles. Since PL-FUT represented a patient friendly method to generate two hair follicles from one hair follicle with consistent results and preserve the donor area, serial application of PL-FUT is suitable for people with progressive scarring alopecia like FFA.

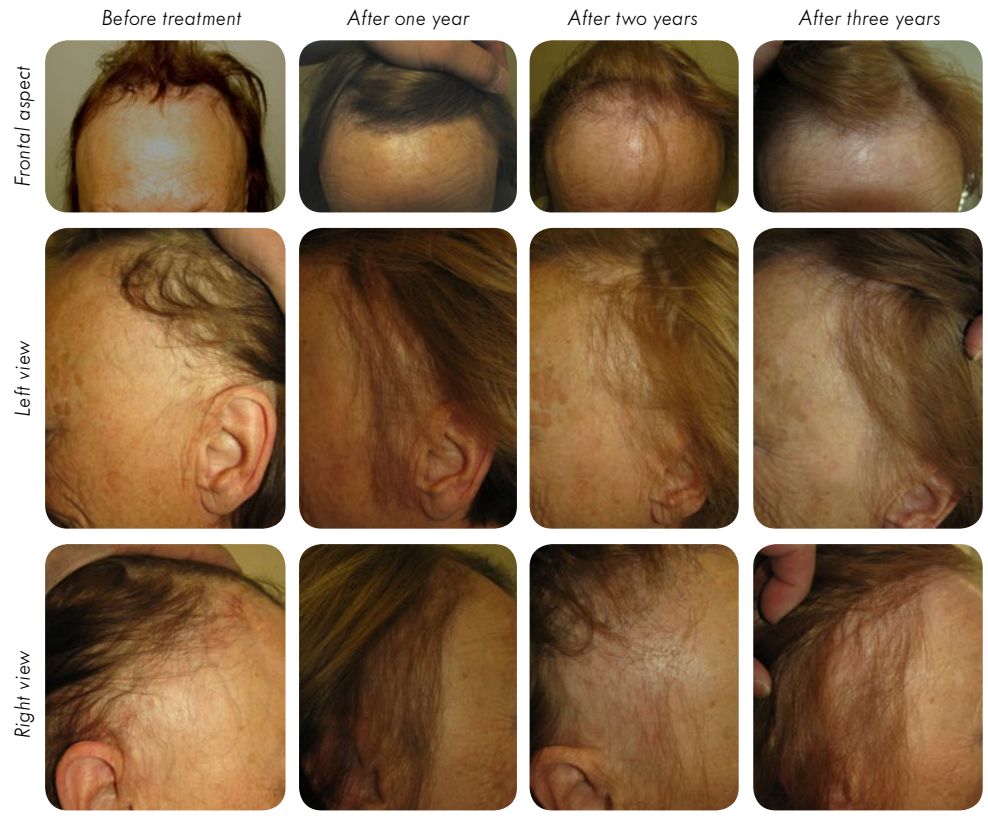
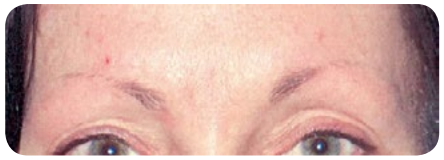
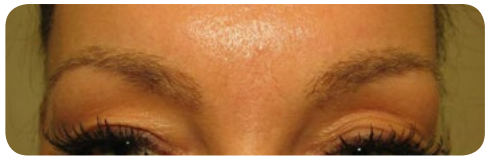


Figure 9: A female patient with Frontal Fibrosing Alopecia (66 years of age at her first treatment), who had a total of 2,952 grafts transplanted spread over 3 consecutive treatments during a period of 3 years in the fronto-temporal area of the scalp and the sideburns. After two years, a part of the implanted hairs of the first treatment had fallen out. However, although some hairs from the first treatment had fallen out after two years and the maximum density had not been achieved, she was satisfied on the patient friendliness of the technique as well as the end result.

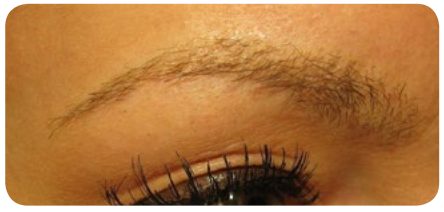
Partial longitudinal follicular unit transplantation (PL-FUT) for the restoration of eyebrows. (Figure 10)
Partial loss of the eyebrows could be the result of epilation, scars as well as inflammatory (skin) diseases like Lupus Erythematosus (LE) or hypothyroidism. Eyebrows played a major role in the expression of the individual feelings, general expressions and emotions.[17,18] Therefore, hair restoration of the eyebrows could be beneficial for the self-esteem of the patient. Since PL-FUT was minimally invasive and the grafts with PL-FUT were much smaller so that they could be placed closer together, a higher density and more natural result could be achieved with PL-FUT for the restoration of eyebrows as compared with traditional hair transplantation techniques.



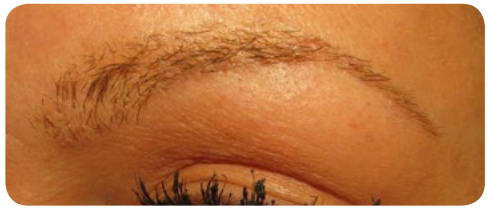
Woman before restoration of the eyebrows.



Woman after restoration of the eyebrows.



Close-up of the right eyebrow.



Close-up of the left eyebrow.

Figure 10: Female patient with partial loss of her eyebrows (37 years of age at her treatment), who had a total of 300 grafts transplanted in one session to restore her eyebrows. The eyebrows were fully restored. Since the eyebrows are very important for the expression of the face, she was very satisfied with the end result.

Limitations of PL-FUT
Although PL-FUT seemed to be an ideal method for the restoration of hair because it is a reliable and patient friendly method to multiply hairs with consistent results and preserve the donor area, the seven years study showed some limitations.

The final and cosmetic result depended not only on the type of grafts (single hair grafts or follicular units), the survival rate of the transplantation and the skills of the surgeon, especially how and where the grafts were placed, but also on the number of grafts which can be transplanted. The human factor was one of the major factors for variability of any (cosmetic) procedure, and thus was also a common problem in hair restoration. Skills and experience of the surgeon as well as that of the technician also varied. A potential risk factor for the loss of density in the donor area was when the grafts were extracted too close to each other. Only qualified and experienced technicians extracted the grafts because smaller grafts were more difficult to extract than complete follicular units in order to minimize the damage during the extraction and to achieve optimal results. The high level of training with continuous evaluation of their skills and the renewal of training contributed significantly in the high rate of extraction of usable grafts, the low complication rate and minimal scarring in the donor area. However, some variability in the regrowth in the donor area were noticed not only between patients but also between different consecutive procedures. There could be several other reasons why re-growth in the donor area varied between patients:

1. Variability in the hair characteristics and the donor skin:

- **A.** Curly hair is more difficult to extract than straight hair.
- **B.** Grey hair is more difficult to extract.
- **C.** Blond hair has smaller follicle units than dark hair and are more difficult to extract.
- **D.** Donor area, which has been used for previous operations with other techniques is more difficult to use as compared with "virgin" donor areas.

2. There were 10-20% of the hair follicles in the donor area that are in the telogen phase, which meant that these follicles were very small. Unfortunately, one was not able to distinguish these telogen hairs from anagen hairs with the eye. The chance of extracting too much tissue from telogen hair follicles to re-generate hairs after extraction was therefore higher than with anagen hair follicles. For example, by extracting 1,400 grafts comprising 2 to 3 hair follicles per graft one could lose 1,400 grafts x 2-3 hairs x 10-20% = 350 to 700 hairs. This could explain that after several treatments some visible density loss appeared.

3. With any consecutive treatment, there were follicles where a part was already extracted. The morphology of these follicles was altered by fibrosis. Therefore, the use of the same follicles was more difficult. So, even with PL-FUT there came an end to the source.

4. The general health condition of the patient was also important. For example, wound healing was decreased in patients who smoked or were diabetic.

In addition, even with PL- FUT one should be realistic about the result – with hair transplantation one can never achieve as full a head of hair as before. However, despite the advantages of PL-FUT described above as compared with those of the traditional hair transplantation, even with PL-FUT a choice must be made on the area one wishes to treat if the recipient area is very large.

Although drugs may achieve good results and the hair loss may be stabilized by the drugs, the result is only temporary.[19] The hair loss will continue once more when the drugs are stopped. Another disadvantage is that drug treatment is only appropriate in the early stages of AGA. The benefits are, on the one hand, the relatively low costs and on the other hand, its non-invasive nature.

Treatment	Area to be treated	Side-effects	Effectiveness	Cost (€) per year	Cost (€) in 10 years
Medicinal - *Topical*					
Minoxidil	Scalp	Allergic reactions, redness, itching, dry skin	Slightly slowing down	300	3,000
Medicinal - *Oral*					
Finasteride	Scalp (esp. crown)	Impotence and reduced libido	Slowing down	550*	5,500
Dutasteride	Scalp (esp. crown)	Impotence and reduced libido	Slowing down	350*	4,200
Cyproterone	Scalp (esp. crown)	Weight change, depression, restlessness, fatigue		200	2,400
(Cimetidine)†	Scalp (esp. crown)	Diarrhea, muscle aches, fatigue, dizziness and 'rash', sometimes also impotence and reduced libido		150	1,800
(Spirono-lactone)†	Scalp (esp. crown)	Diarrhea, muscle aches, fatigue, dizziness and 'rash', sometimes impotence and reduced libido		100*	1,200
Surgical					
Strip method	Scalp NH III-IV (1 treatment)	Pain, scar	Lasting result. However hair loss if not treated		5,000
	Scalp NH V-VI (2 treatments)	Pain, scar	Lasting result. However hair loss if not treated		10,000
Follicul unit extraction	Scalp NH III-IV (1 treatment)	Scars	Lasting result. However hair loss if not treated		6,000
	Scalp NH V-VI (2 treatments)	Scars	Lasting result. However hair loss if not treated		12,000
PL-FUT	Scalp & Face NH III-IV (1 treatment)	None	Lasting result. However hair loss if not treated		6,000
	Scalp & Face NH V-VI (2 treatments)	None	Lasting result. However hair loss if not treated		12,000
Hair work					
Extensions	Scalp	Broken hairs	Full head of hair is However, hair loss if not treated.	600§	6,000
Full/Fusion	Scalp	Allergic reaction to the suture material	Full head of hair is However, hair loss if not treated.	1,250§	12,500

One should also be realistic about the result of surgical treatment – with hair transplantation one can never achieve as full a head of hair as before.

In our previous studies we revealed PL-FUT has great advantages over the traditional hair transplantation techniques, namely:

- Preservation of donor hair follicles, no scars and no stitches and the result of which is not visible on the back of the head after grafts were removed.
- No loss of density because the remaining hair follicles will continue to produce new hairs.
- Almost no pain (both during as well as after the treatment) and better and faster healing of wounds because PL-FUT is minimally invasive.
- A higher density and more natural result with PL-FUT than that with traditional hair transplantation techniques because the grafts with PL-FUT are much smaller so that they can be placed closer together.

The greatest weakness of the published articles is the number of patients evaluated, so that no stastistics could be performed. However, in view of the fact that each graft is actually an individual test, because otherwise no regrowth is visible, I believe it is still possible to draw the above conclusions.

Although surgical treatments are also expensive, they give a cosmetically good and lasting result (Figure 6). However, transplantation techniques have no effect on the primary process. In order to halt AGA's progression after transplantation, it may be decided to additionally treat AGA with medication.

Table 1: Advantages and disadvantages of the most common treatments for androgenetic alopecia.

NH Type according to Norwood-Hamilton, PL-FUT = Partial longitudinal follicle unit transplantation.

* *According to Health Care Insurance Board (CVZ).*

† *Rarely prescribed and with low effectiveness.*

‡ *Sometimes, with Norwood-Hamilton type III-IV, a follow up treatment is necessary in order to treat the progression of alopecia. With 1 or 2 treatments, approx. 1,000 or 2,000 "grafts" (2,500 or 5,000 hairs) respectively are transplanted.*

§ *On the basis of one synthetic hair work per year including maintenance costs.*

Treatment of patients with androgenetic alopecia is aimed at camouflage (hair work), growth stimulation (Minoxidil), hormonal interference (Finasteride, Dutasteride or Cyproterone), or takes the form of a surgical procedure (Strip method, Follicle unit extraction or Partial longitudinal follicle unit transplantation (PL-FUT).

The main advantage of hair prosthesis is the cosmetically satisfactory and immediate result. Moreover, it is (partially) paid by most health insurance companies. However, it remains a substitute and a hair work is usually a very laborious item and it requires regular maintenance, and thus cost inefficient on the longterm.[20] Young people in the early stages of AGA benefit from drug treatment when one wishes to halt the process. However, if a full head and an immediate result are desired, then a hairpiece is an option. At an advanced stage of AGA, a permanent result can only be achieved with a hair transplantation. Sometimes a follow up treatment is necessary to treat the progression of AGA. However, a well-performed transplantation takes this into account by also transplanting hairs between the existing hair growth so that the regression is much less visible. Wwhen one wishes to temporarily slow or stabilize the process, a treatment with drugs is cost-effective in the short-term (one year) and medium-term (10 years). In the long-term (more than 10 years), drug treatment will not outweigh the costs, because the result of the drugs is only temporary. From this point of view, hair transplantation, even if a further treatment is necessary, will be the best solution because it provides a permanent result. Since AGA is a cosmetic problem, the voice of the patient will weigh heavily. Good information is essential. Table 3 summarizes the most common treatments with pros and cons and the costs for the patient. If there are major psychological problems related to the AGA, a psychologist should be consulted before the treatment. Our experience is that appropriate advice given in the context of such a careful policy clearly enhances the quality of life for most patients.

Hair follicular stem cells for exploring their potential future applications in regenerative medicine.

In this study, it was demonstrated that stem cells from hair follicles (HFs) plucked from human scalps show a NCSC immune profile and can be cultivated, expanded and kept frozen until required. It was also shown that differentiation of these stem cells into neurons and glial cells is feasible.

The technique used to harvest the HFs is virtually painless and allows the collection of hundreds of HFs per patient. The culture method to expand the cells is simple and straightforward and usually yields 1x 10^4 cells per HF. Only three to four passages are required for this high yield, so cellular senescence is not an issue. Moreover, cellular damage by frequent protease treatments is limited to a minimum. Altogether, the character of hair follicle bulge stem cells (HFBSCs), the easily accessible area of harvesting and the straightforward upscaling of the cells, make HFBSCs very attractive candidates for cell-based therapies. However, there are some critical points in the procedures such as the selection of intact hair follicles, rejection of cultures with undesired cell outgrowth and establishing the micro-environment necessary for targeted differentiation.

Our culture studies showed that stem cells are prone to changes, and this is their nature . They adapt quickly to differences in circumstances such as changes in the medium composition or extra cellular matrix (ECM). This can be both an advantage or a disadvantage since the slightest changes in culture conditions may result in great (mostly undesired) effects. We now realize that there are seven major factors, which determine the fate of stem cells. The type of stem cells (pluri-, multi-, unipotent), the origin of the stem cells (meso-, ecto- or endodermal), the medium composition, the ECM, the culture dimensions (2D versus 3D), the temperature and the extra additives (e.g. vitamin A).

Statistics teaches us that with so many variables, the desired outcome is highly unpredictable. In the studies described in this thesis, we therefore cultured under strictly controlled conditions (standardized operation procedures) and performed critical observations and controlled analyses of the cultured cells. In doing so, we believe that we gradually obtained an insight into the major factors mentioned above.

This is clearly illustrated in the Figure 11, which shows that in the same culture, different types of (stem) cells grow out from different parts of the HF. We now recognize that each population of (stem) cells needs a specific treatment in order to achieve optimal proliferation and differentiation. As a consequence, in our new experiments, we culture different parts of the HF separately.

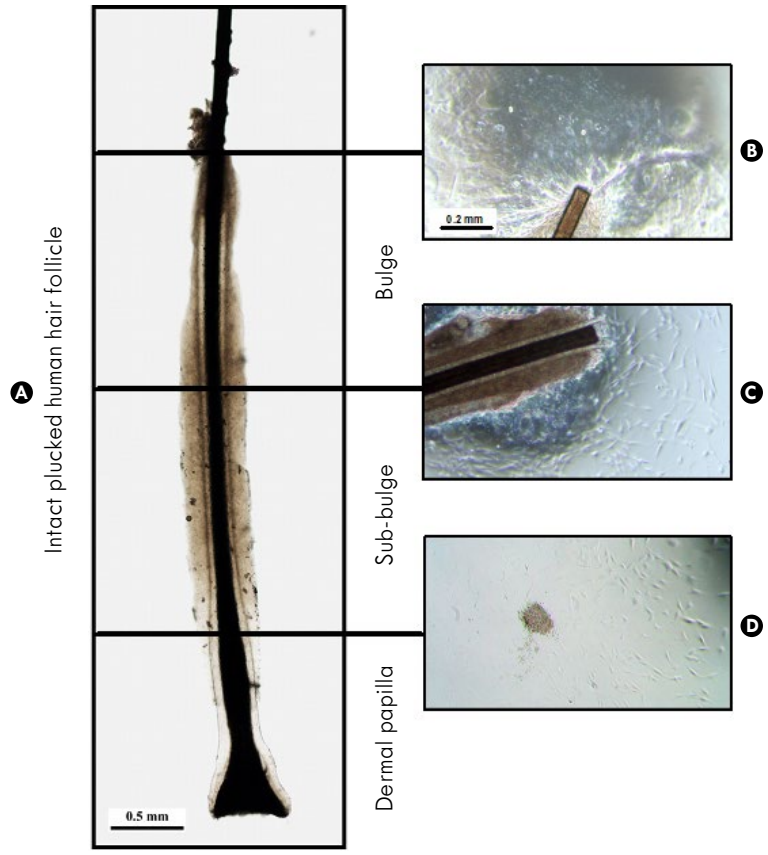


Figure 11: Phase contrast images of different types of cells, which have migrated from different parts of the human HF after 12 days of culture under standard culture conditions as reported by Gho et al, 2016.[24] **(A)** *different parts of the HF are indicated.* **(B)** *Massive outgrowth of cells from the bulge region, characterized as keratinocytes by their polyhedral morphology.* **(C)** *Cells from the sub bulge region, three different types of cells are recognized: keratinocytes (arrow), fibroblasts (arrowhead) and neural crest cells (*).* **(D)** *Disintegrated dermal papilla and migrating fibroblasts.*

In our in vitro and in vivo studies we also noticed that the ECM components are crucial for survival and outgrowth of cells from HFs (derived from plucked hairs). This knowledge is highly important for our studies on regeneration and multiplication of hair follicles. For this reason we decided to test different matrices and the first results are shown in Figure 12.

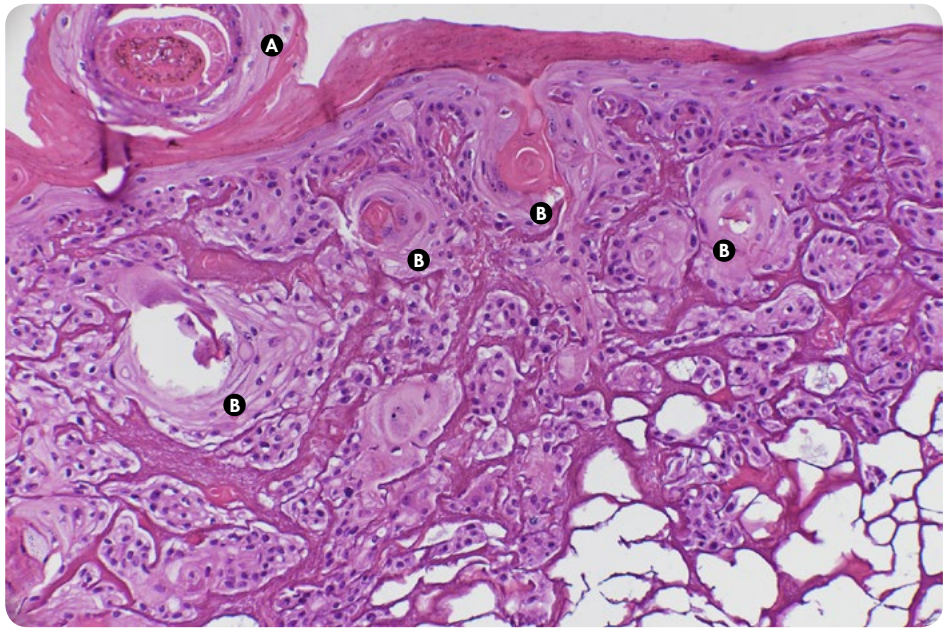


Figure 12: This picture shows a plucked hair from the author **(A)**, *cultured with Novomaix® (Matricel, Germany) as ECM. The culture method of Coolen et al. was used.[25] After 21 days of culture at 37°C, numerous cells migrated into the matrix and some hair germ-like structures* **(B)** *were formed.*

Follicular stem cells in regenerative medicine, future perspectives.

The virtually painless method to obtain hair follicles from the scalp of patients and the potency of the stem cells residing in the hair follicle opens a way for cell-based therapy in a variety of diseases. Moreover, the hair follicle is a relatively immune-privileged zone[21], which makes the HF stem cells not only potential candidates for autologous-, but also for allogeneic stem cell-based treatment.

However, it is clear that prior to clinical application of cultured hair follicular stem cells, the major challenge will be to find the ideal combination of the critical factors mentioned above, among others. medium, additives and ECM to direct the variety of cells from the HF towards an appropriate phenotype. Based on the in vivo and in vivo results, we recommend that after isolation of HFs, the tissue drenched in appropriate medium should be placed in a 3D matrix as soon as possible. This can be the patient's skin or a 3D commercially available ECM. Besides further characterization of the HF stem cells, it is also necessary to investigate the reciprocal effects of (stem) cells from various parts of the HF. It should be investigated which type(s) of cells are crucial to produce hair germ-like structures[22] and subsequently new hairs. In our opinion, this knowledge would lead to the development of a new method to multiply hair.

Nevertheless, according to the author's view, the future towards the use of (cultured) follicular stem cells derived from plucked hair to regenerate new hair (follicles) is a matter of time. The use of such follicular stem cells for other clinical applications is more challenging, because it is possible that some stem cell populations from hair follicles may follow their innate biological inclination when used for cell-based therapies in organs other than the skin.[23] Hence, a careful selection of the desired type of stem cells is a prerequisite in such a situation.

During the following years we will focus on the development of an appropriate ECM for 3D HF stem cell culture and examine the option of transplanting cultured follicular stem cells obtained from different areas of the hair follicle. The most suitable ECM will be combined with follicular stem cells to support the survival and the development of the transplanted cells.

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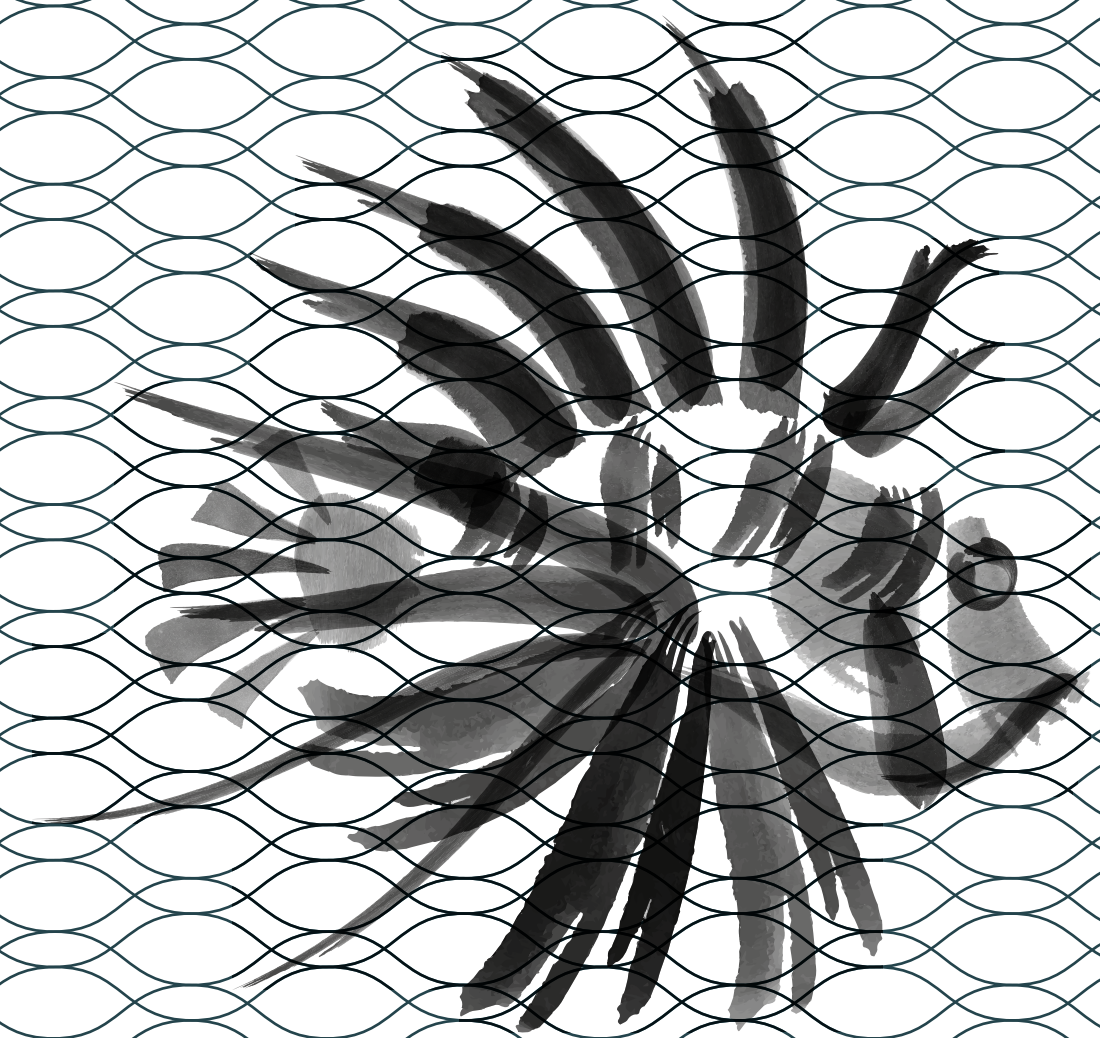


7

SUMMARIES

7.1 ENGLISH SUMMARY

A general introduction of this thesis is presented in Chapter 1. The anatomy and the physiology of the hair are clarified and the different types of hair loss are dealt with. Particular attention has been paid to Androgenetic Alopecia (AGA), which is the most common type both in men and in women and explain the topical, the oral and the surgical treatment modalities in AGA. At present, the surgical treatment is the only treatment with permanent results. Since the follicular stem cells may play a crucial role in hair restoration by hair transplantation, the current scientific status of the follicular stem cells is also dealt with.

Therefore, the main objectives of this thesis were to identify and determine the localization of the follicular stem cells in whole hair follicles, plucked hairs and hair follicular cell culture; to study the viability of hair transplantation grafts and hair follicular units; to explore the hair growth potential of partial follicular units containing follicular stem cells and their clinical applications and evaluate the results as well as to study hair follicular stem cells and explore their potential future applications in regenerative medicine.

The possibility to use the hair follicle cells attached to plucked hair for follicle cell culture was examined in studies described in Chapter 2. These follicular cells were phenotyped in an attempt to identify the stem cell fraction. Reports in the literature indicated that this cell population may be positive for cytokeratin (CK) 19. Since stem cells in general need to be protected from apoptosis, the presence of the apoptosis-suppressing Bcl-2 protein together with the absence of the apoptosis-promoting Bax and the CK profile were used as an indicator of the stem cell population in the hair follicle and in cultures of hair follicle cells. Co-expression for CK19 and Bcl-2, but not Bax was observed in two distinct areas localized in the upper and lower third of the follicle from both skin biopsies and plucked hairs, whereas proliferation markers were absent in these areas. Cytokeratin 19 and Bcl-2 were also co-expressed in combination in a fraction of the follicular cell culture. The skin basal cell marker CK14 was not detected throughout the outer root sheath of the hair follicle from both skin biopsies and plucked hairs as well as in the follicular cell culture.

Since CK19 / Bcl-2-positive and Bax-negative cells could be obtained from cells derived from plucked hair and were retained in the cultures from these cells, our finding endorsed the assumption that stem cells were located in the bulge area of the hair follicle because they were not found in or near the dermal papilla.

The importance of the preservation solution on the viability of hair transplantation grafts and hair follicle units is described in Chapter 3. Hair transplantation techniques have changed in the last decades since the introduction of follicular unit extraction. Since the size of the grafts is currently much smaller than in the past, these grafts are also much more vulnerable. Grafts harvested from the donor area are devoid of blood supply and thus also from oxygen and nutrients. In the studies described in this chapter, we examined the influence of the preservation solution on the viability of the grafts. One of the main ingredients of the preservation solution was Bis(maltolato)oxovanadium (BMOV). This compound was shown to inhibit tyrosine phosphatase leading to an increased tyrosine phosphorylation state. This phosphatase inhibition could have been protective against apoptosis, even when it was used late after the onset of lack of oxygen as in re-perfusion damage.

The influence of two commercially available preservation media, saline solution (Braun, Melsungen, Germany) and Ringer's lactate (Braun), on the viability of grafts were investigated and were compared with the preservation solution developed by Hair Science Institute which contained BMOV, with trypan blue as vital staining technique. The grafts stored in the preservation solution developed by Hair Science Institute showed a significantly better viability as compared with the two commercially available preservation media saline solution and Ringer's lactate. The results of the study showed that a preservation solution could influence the viability of the grafts, which would be essential for hair transplantations with small grafts. This solution could make transplants with partial longitudinal follicular unit grafts possible and successful.

The hair regrowth potential of partial longitudinal follicular units (PL-FU's) and their clinical applications in hair restoration are dealt with in Chapter 4. It was observed that transplanted PL-FUs, which contained sufficient viable follicular stem cells as a result of the modified preservation solution, to the recipient area could be used as complete follicular units to regenerate completely differentiated hair growth with the same characteristics as in the donor area, but only the number of hairs in the new follicle may be lower than the average. It was also noted that the partial follicular units in the donor area could survive and produce the same number of hairs with the same characteristics. Partial Longitudinal follicular unit transplantation (PL-FUT) may represent the first reliable patient friendly method to generate two hair follicles from one hair follicle with consistent results and preservation of the donor area with almost no scarring.

Besides AGA, PL-FUT is highly suitable for people with facial and/or scalp burns. In the studies, treated burn victims had satisfactory or highly satisfactory cosmetic results.

Even facial hair restoration of the eyebrows with PL-FUT in healthy individuals and in burn scar tissue improved the appearance and the psychological well-being of the patients in a reliable and patient friendly way.

To evaluate the long-term efficacy and results of PL-FUT, a retrospective single center study between 2005 to 2011 was performed in 4,819 hair loss patients from whom 1,983 (41.1%) patients were treated with 2,646 treatments in total of which 1,983 (74.9%) were initial and 663 (25.1%) consecutive treatments. There were 2,306 (87.1%) treatments evaluated of which 2,202 (95.5%) were considered satisfactory according to the patients after one year. In the donor area, there was no visible density loss in 2,276 (98.7%) and some density loss in 30 (1.3%) of the patients.

In this study, PL-FUT was shown to be an effective technique for androgenic and cicatricial alopecia with a high patients satisfaction, minimal side effects, natural result and preservation of the donor area.

The growth potential of follicular stem cells and their future applications in regenerative medicine is the focus of studies described in Chapter 5. Stem cells from the adult hair follicle bulge may differentiate into neurons and glia, which is advantageous for the development of an autologous cell-based therapy for neurological diseases. Consequently, bulge stem cells from plucked hair may increase opportunities for personalized neuro-regenerative therapy. In the studies, we observed that stem cells derived from plucked hairs had neural crest stem cells characteristics. They were easily be cultivated, expanded and kept frozen until needed while keeping NCSC characteristics. Therefore, these stem cells allow practical application of hair follicle stem cells as a source for regenerative medicine.

A general discussion of the studies performed in this thesis and the reported studies by others is presented in Chapter 6. Both the scientific and the clinical applications of our findings, especially the Partial Longitudinal follicular unit transplantation have been highlighted. Both the possibilities and the limitations of the follicular stem cells in regenerative and cosmetic medicine are also dealt with.

7.2 NEDERLANDSE SAMENVATTING

Een algemene inleiding van dit proefschrift wordt gepresenteerd in Hoofdstuk 1. De anatomie en de fysiologie van het haar worden uitgelegd en de verschillende soorten haaruitval worden besproken. Er is speciale aandacht besteed aan Androgenetische Alopecie (AGA), het meest voorkomende type haaruitval bij zowel mannen als vrouwen. Daarnaast wordt er uitleg gegeven van de actuele, de orale en de chirurgische behandelingsmodaliteiten bij AGA. Op dit moment is de chirurgische behandeling de enige behandeling met blijvende resultaten. Omdat de folliculaire stamcellen een cruciale rol kunnen spelen bij haarrestoratie door haartransplantatie, wordt ook de huidige wetenschappelijke stand van zaken van de folliculaire stamcellen behandeld.

De hoofddoelstellingen van dit proefschrift waren het identificeren en bepalen van de lokalisatie van de folliculaire stamcellen in hele haarfollikels, geplukte haren en celculturen uit haarfollikels; de levensvatbaarheid van haartransplantatie-grafts en haar folliculaire units te bestuderen; om het haargroeipotentieel van partiële folliculaire eenheden met folliculaire stamcellen en hun klinische toepassingen te onderzoeken en de resultaten te evalueren alsmede haar folliculaire stamcellen te bestuderen en hun potentiële toekomstige toepassingen in de regeneratieve geneeskunde te onderzoeken.

De mogelijkheid om de folliculaire cellen te gebruiken die gehecht zijn aan geplukt haar voor de folliculaire celcultuur, werd onderzocht in studies beschreven in Hoofdstuk 2. Deze folliculaire cellen werden gefenotypeerd in een poging de stamcelfractie te identificeren. Rapporten in de literatuur wezen erop dat deze stamcelpopulatie positief zou moeten zijn voor cytokeratine (CK) 19. Aangezien stamcellen in het algemeen moeten worden beschermd tegen apoptose, is de aanwezigheid van het apoptose-onderdrukkende Bcl-2-eiwit samen met de afwezigheid van de apoptose-stimulerende Bax en het CK-profiel, gebruikt als een indicator van de stamcelpopulatie in de haarfollikels en in culturen van haarfollikels. Co-expressie voor CK19 en Bcl-2, zonder expressie van Bax werd waargenomen in twee afzonderlijke gebieden gelokaliseerd in het bovenste en onderste derde deel van de follikel van zowel huidbiopten als geplukte haartjes, terwijl proliferatiemarkers afwezig waren in deze gebieden. Cytokeratine 19 en Bcl-2 kwamen ook gezamenlijk tot expressie in een deel van de folliculaire celkweek. De basale celmarker van de huid CK14 werd niet gedetecteerd over de gehele de buitenste wortelschede van de haarzakjes van zowel huidbiopten en geplukte haren als in de folliculaire celcultuur.

Omdat CK19 / Bcl-2-positieve en Bax-negatieve cellen konden worden verkregen uit cellen die waren geoogst van geplukt haar en deze ook in de kweken van deze cellen aantoonbaar waren, bevestigde onze bevinding de aanname dat stamcellen zich in het bulge gebied van de haarzakjes bevonden omdat ze niet werden gevonden in of nabij de dermale papilla.

Het belang van de preservatie-oplossing voor haartransplantatie grafts en haarfollikels wordt beschreven in hoofdstuk 3. Haartransplantatietechnieken zijn in de laatste decennia

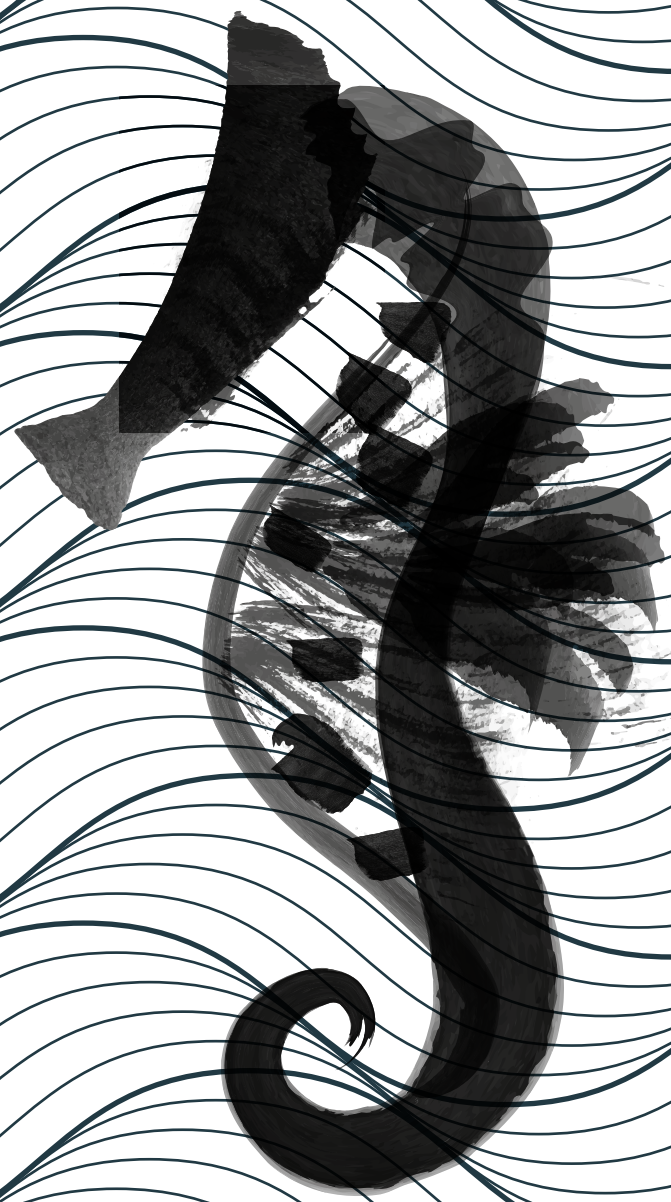
sinds de introductie van folliculaire unit extractie veranderd. Omdat de grootte van de grafts momenteel veel kleiner is dan in het verleden, zijn deze grafts ook veel kwetsbaarder. Grafts geoogst uit het donorgebied zijn verstoken van bloedtoevoer en dus ook van zuurstof en voedingsstoffen. In de studies die in dit hoofdstuk worden beschreven, hebben we de invloed van de preservatie oplossing op de levensvatbaarheid van de grafts onderzocht. Een van de hoofdingrediënten van de conserveringsoplossing was Bis(maltolato)oxovanadium (BMOV). Van deze verbinding werd aangetoond dat deze tyrosinefosfatase remt, wat leidt tot een verhoogde tyrosinefosforylatie toestand. Deze fosfatase-inhibitie zou beschermend kunnen zijn geweest tegen apoptose, zelfs wanneer deze laat werd gebruikt na het begin van gebrek aan zuurstof zoals bij re-perfusieschade.

De invloed van twee in de handel verkrijgbare conserveermiddelen, zoutoplossing (Braun, Melsungen, Duitsland) en Ringer's lactaat (Braun) op de levensvatbaarheid van grafts werden onderzocht en vergeleken met de bewaaroplossing ontwikkeld door Hair Science Institute met BMOV, met trypan blauw als vitale kleuringstechniek. De grafts die waren opgeslagen in de preservatie soplossing ontwikkeld door Hair Science Institute vertoonden een significant betere levensvatbaarheid in vergelijking met de twee in de handel verkrijgbare preservatie middelen zoutoplossing en Ringer's lactaat. De resultaten van het onderzoek toonden aan dat een preservatie oplossing de levensvatbaarheid van de grafts zou kunnen beïnvloeden, wat essentieel zou zijn voor haartransplantaties met kleine grafts. Deze oplossing zou transplantaties met partiële longitudinale follikels mogelijk en succesvol kunnen maken.

Het haargroeipotentieel van partiële longitudinale folliculaire units (PL-FU's) en hun klinische toepassingen bij haartransplantatie worden behandeld in Hoofdstuk 4. Er werd waargenomen dat getransplanteerde PL-FU's, die voldoende levensvatbare folliculaire stamcellen bevatten als gevolg van de gemodificeerde preservatie oplossing, in het ontvangstgebied kunnen worden gebruikt als complete folliculaire units om volledig gedifferentieerde haargroei te regenereren met dezelfde kenmerken als in het donorgebied. Alleen het aantal haren in de nieuwe folliculaire unit zou lager kunnen zijn dan het gemiddelde. Er werd ook opgemerkt dat de achtergebleven partiële folliculaire units in het donorgebied konden overleven en hetzelfde aantal haren met dezelfde kenmerken konden produceren. Partiële longitudinale folliculaire unit transplantatie (PL-FUT) kan de eerste betrouwbare patiëntvriendelijke methode zijn om twee haarzakjes van één haarzakje te genereren met consistente resultaten en behoud van het donorgebied met bijna geen littekens. Naast AGA is PL-FUT uitermate geschikt voor mensen met gezichts- en / of hoofdhuidverbranding. In de onderzoeken hadden behandelde slachtoffers van brandwonden bevredigende of zeer bevredigende cosmetische resultaten. Zelfs restoratie van wenkbrauwen bij gezonde personen en bij littekens ten gevolge van verbranding door middel van PL-FUT verbeterden het uiterlijk en het psychisch welbevinden van de patiënten in een betrouwbare en patiëntvriendelijke manier. Om de werkzaamheid op lange termijn en de resultaten van PL-FUT te evalueren, werd een retrospectieve single center-studie tussen 2005 en 2011 uitgevoerd bij 4.819 patiënten met haarverlies bij wie 1.983 (41.1%) patiënten werden behandeld met 2.646 behandelingen in totaal waarvan 1.983 (74,9%) initiële en 663 (25,1%) opeenvolgende behandelingen waren. Er werden 2,306 (87,1%) behandelingen geëvalueerd, waarvan 2.202 (95,5%) na één jaar als bevredigend werden beschouwd volgens de patiënten. In het donorgebied was er geen zichtbaar dichtheidsverlies bij 2.276 (98,7%) en enig dichtheidsverlies bij 30 (1,3%) van de patiënten. In dit onderzoek bleek PL-FUT een effectieve techniek te zijn voor androgene en verlittekende alopecia met een hoge patiënten tevredenheid, minimale bijwerkingen, natuurlijk resultaat en behoud van het donorgebied.

Het groeipotentieel van folliculaire stamcellen en hun toekomstige toepassingen in regeneratieve geneeskunde is de focus van studie beschreven in Hoofdstuk 5. Stamcellen uit de bulge gebied van volwassen haarfollikels kan differentiëren in neuronen en glia, wat gunstig is voor de ontwikkeling van een autologe stamcel therapie voor neurologische aandoeningen. Als gevolg daarvan kunnen stamcellen uit de bulge gebied van geplukte haren de mogelijkheden voor gepersonaliseerde neuro-regeneratieve therapie vergroten. In ons onderzoek hebben we waargenomen dat stamcellen van geplukte haren eigenschappen hebben van neurale lijst stamcellen (NCSC). Ze werden gemakkelijk gecultiveerd, uitgebreid en bevroren gehouden tot ze nodig waren, terwijl de NCSC-kenmerken behouden bleven. Daarom laten deze stamcellen praktische toepassing toe van stamcellen van haarfollikels als een bron voor regeneratieve geneeskunde.

Een algemene bespreking van de onderzoeken die in dit proefschrift en gerapporteerde onderzoeken door anderen zijn uitgevoerd, wordt gepresenteerd in hoofdstuk 6. Zowel de wetenschappelijke als de klinische toepassingen van onze bevindingen, in het bijzonder de partiële longitudinale folliculaire unit transplantatie (PL-FUT), zijn benadrukt. Zowel de mogelijkheden als de beperkingen van de folliculaire stamcellen in regeneratieve en cosmetische geneeskunde komen ook aan bod.



8

APPENDICES

8.1 ABBREVIATIONS

AGA	Androgenetic Alopecia
ANOVA	Analysis Of Variance
ATP	Adenosine TriPhosphate
BCC	Basal Cell Carcinoma
Bcl	B-cell lymphoma
BGM	Basic Growth Medium
BIO-GAM	Biotinylated Goat–Anti-Mouse
BIO-GAR	Biotinylated Goat–Anti-Rabbit
BMOV	Bis(maltolato)-oxovanadium
cAMP	3'-5'-cyclic Adenosine MonoPhosphate
CAO	Coronary Artery Occlusion
CK	CytoKeratine
CVZ	Health Care Insurance Board
DAPI	4´,6- iamidino-2-phenylindole
DHT	DiHydroTestosterone
DMEM	Dulbecco's Modified Eagle Medium
dSFK	Defined Serum-Free Keratinocyte Growth Medium
ECM	Extra Cellular Matrix
EDF	European Dermatology Forum
EDTA	Ethylene DiamineTetra-acetic Acid
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FFA	Frontal Fibrosing Alopecia
FITC	Fluorescein IsoThioCyanate
FUE	Follicle Unit Extraction / Follicle Unit Excision
FOV	Field of View
HE	Haematoxylin Eosin
HF	Hair Follicle
HFBSC	Hair Follicle Bulge-derived (Neural Crest) Stem Cells
HFSC	Hair Follicle Stem Cells
HSI	Hair Science Institute
iPSC	Induced Pluripotent Stem Cells
KATP	ATP-sensitive Potassium
NCSC	Neural Crest-derived Stem Cells
ORS	Outer Root Sheath
PDGF	Platelet-Derived Growth Factor
PDL	Poly-D-Lysine
PCO	Potassium (K+) Channel Opener.
PL-FUT	Partial Longitudinal - Follicular Unit Transplantation
PBS	Phosphate Buffered Saline
TRITC	TetramethylRhodamine IsoThioCyanate

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8.3 LIST OF PUBLICATIONS

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8.4 CURRICULUM VITAE

Coen Gho was born on May 13th, 1967 in Jakarta, Indonesia to Gho Tjeng Hoei and Magdalena Gho-Lauw. He has two brothers, Albert and Ben, who are both medical doctors as well. In 1971, the whole family moved from Indonesia to the Netherlands where the first lived in 's-Hertogenbosch. In 1973, they moved to 's-Gravenzande, where he went to elementary school and college. When he was 18, he started his medical study at the Erasmus University in Rotterdam, the Netherlands. He currently lives in Maastricht, the Netherlands and works as a specialist on hair- and scalp diseases at Hair Science Institute at different locations in Europe, Middle East and Asia. Besides his work in the field of hair restoration, he is also involved in fundamental hair research and clinical studies since 1992 in close collaboration with universities and (academic) hospitals. Besides developing several new therapies and treatment methods in the field of hair diseases and burns, he also studies and develops new physiological concepts and therapies in the field of cardiology, neurodegenerative diseases and burns.

He lives with his wife, Sheila Njo, who is better looking and a better person in general. They have met during their studies through their parents. They have a wonderful son and lovely daughter, Sylvian and Caitlyn. He plans to continue to improve his humanly, clinical and scientific skills.

8.5 PHD PORTFOLIO

Presentations

1997, 18th International Society of Dermatological Surgery Meeting, Amsterdam, the Netherlands. Hair-Restoration by Hair Multiplication. Coen G.Gho.

2001 August 22-26, 16th European Cytoskeleton Forum Meeting, Maastricht, the Netherlands. Preservation of phenotype in plucked hair and cultured follicular keratinocytes - Poster. Coen G.Gho, Jacqueline E.F.Braun, Claudia M.L.J. Tilli, Frans C.S. Ramaekers, H.A. Martino Neumann.

2002, August 11-16, 11th Quadrennial Congress of the International Society for Burn Injuries, Seattle, WA, USA, (Abstract 360) Efficacy of bis(maltolato)oxovanadium (BMOV) in a contact burn model in pigs - Poster. Gho C. et al.

2003, October 15-19, 11th International Society of Hair Restoration Surgery Meeting, New york, USA. To Multiply or not to Multiply, that's the question. Coen G.Gho.

2006, June 8, Cosmoderm VIII Meeting, Rotterdam, NL. Follicle by Follicle / HairStemcell Transplantation. Coen G.Gho.

2007, March 23, Anti-Aging Medicine World Congress 2007, Monte Carlo, Monaco. Human Follicular Stem cells : Today and tomorrow. Coen G.Gho.

2007, May 16, European Academy of Dermato-Venerology Congress 2007, Vienna, Austria. Hair Stemcell Transplantation. Coen G.Gho.

2008, September 5, International Society of Hair Restoration Surgery (ISHRS) Congress 2008, Montreal, Canada. Hair Stemcell Transplantation. Coen G.Gho.

2009, July 25, International Society of Hair Restoration Surgery (ISHRS) Congress 2009, Amsterdam, the Netherlands. Hair Stemcell Transplantation – Possibilities & Limitations. Coen G.Gho.

2010, September 23, International Society of Dermatology (ISDS) Congress 2010, Bucharest, Rumania . Partial Longitudinal - Follicular Unit Transplantation – A method to optimize hair transplantation. Coen G.Gho.

2011, April 8, International Society of Dermatology (ISDS) Congress 2011, Bali, Indonesia. Current treatment options for managing male pattern baldness. Coen G.Gho

2012, September 14, Aesthetic Asia 2012, Singapore, Singapore. HairStemcell Transplantation (HST) - Partial Longitudinal Follicular Unit Transplantation (PL-FUT). Coen G.Gho

2012, September 12, 16th Congress of International Society for Burn Injuries, Edinburgh, United Kingdom. Improved hair restoration method for burns. Coen G.Gho.

2012, October 4, 22nd European Tissue Repair Society Meeting 2012, Athens, Greece. Hair restoration by Partial Longitudinal Follicular Unit Transplantation (PL-FUT) - Poster. Coen G.Gho.

2012, October 5, International Master Course on Aging Skin (IMCAS) Asia 2012, Hong Kong, Hong Kong. Partial Longitudinal - Follicular Unit Transplantation – A method to optimize hair transplantation. Coen G.Gho.

2013, January 18, 31st Jahrestagung der Deutschsprachigen Arbeitsgemeinschaft für Verbrennungsbehandlung 2013, Mayrhofen, Austria. Improved hair restoration method for burns. Coen G. Gho.

2013, August 25, 7th AustralAsian Academy of Anti-Aging Medicine Conference 2013, Melbourne, Australia. Partial Longitudinal - Follicular Unit Transplantation (PL-FUT) - A method to optimize hair transplantation. Coen G. Gho.

2013, August 30, 1st Education meeting Hair diseases 2013, Maastricht, the Netherlands. Follicular stem cells. Coen G. Gho.

2013, September 10th, 50th Inner Ear Biology, Madrid, Spain. Plucked human scalp hair follicles may serve inner ear cell-based therapy. Coen G. Gho, Margriet A. Huisman.

2013, September 12, Aesthetic Asia 2013, Singapore, Singapore. Partial Longitudinal Follicular Unit Transplantation (PL-FUT) – A new era in hair restoration. Coen G.Gho

2013, November 9, 2nd DASIL Meeting 2013, Bangkok, Thailand. A New Approach to Hair Transplantation. Coen G. Gho.

2014, August 29, 14th Indonesian Society of Dermatology and Venereology National Congress Bandung, Indonesia. Hair follicle as source of stem cells. Coen G. Gho.

2014, September 8, 3rd DASIL Meeting 2014, Sun City, South Africa. The treatment of Cicatricial Alopecia with Partial Longitudinal-Follicular Unit Transplantation. Coen G. Gho.

2014, September 9, 3rd DASIL Meeting 2014, Sun City, South Africa. Follicular Stem cell Transplantation. Coen G. Gho.

2014, October 10, 23rd European Academy of Dermatology and Venereology (EADV) Congress, Amsterdam , The Netherlands. The principles of hair transplantation. Coen G. Gho.

2015, July 31st, International Master Course on Aging Skin (IMCAS) Asia 2015, Bali, Indonesia. The principles of hair transplantation. Coen G.Gho

2015, August 1st, International Master Course on Aging Skin (IMCAS) Asia 2015, Bali, Indonesia. The treatment of cicatricial alopecia with Partial Longitudinal – Follicular Unit Transplantation (PL-FUT). Coen G.Gho.

2015, August 2nd, International Master Course on Aging Skin (IMCAS) Asia 2015, Bali, Indonesia. Follicular stem cells in cosmetic and regenerative medicine. Coen G.Gho.

2016, September 16, 37th Annual Congress of the International Society for Dermatologic Surgery (ISDS), Amsterdam, the Netherlands. Follicular stem cells in regenerative medicine. Coen G.Gho.

2016, November 26th, International Congress of Aesthetic Dermatology (ICAD), Bangkok, Thailand. Follicular stem cells in regenerative and cosmetic medicine. Coen G.Gho.

2017, January 26th, International Master Course on Aging Skin (IMCAS) World Congress 2017, Paris, France. Follicular Stem Cells In Cosmetic & Regenerative Medicine. Coen G.Gho.

2017, November 15th, Regenerative Medicine and Stem cells World Congress 2017, Singapore, Singapore. Follicular Stem Cells In Cosmetic & Regenerative Medicine. Coen G.Gho.

2017, December 8th, Dutch Society for Cosmetic Medicine Congress 2017, Utrecht, The Netherlands. Stem Cells In Cosmetic & Regenerative Medicine. Coen G.Gho.

Patent (application)s & Intellectual Property

1997, March 5th. Method for the propagation of hair
A method for the cosmetic reproduction of hair, which method comprises:(a) plucking hair in the anagen phase from one or more donor regions (b) culturing keratinocytes from the hair removed under conditions whereby the keratinocytes multiply, wherein the keratinocytes are cultured in a culture medium supplemented with (i) at least one human mast cell line and/or autologous cultured CD34cells, or (ii) one or more extracts of a human mast cell line(s) and/or of the autologous CD34cells, and/or (iii) growth-stimulating agents; and (c) introducing the cultured keratinocytes into the pores of receptor regions.

1997, July 29th. Use of physiologically acceptable vanadium compounds, salts and complexes.
A method of prophylactic treatment of secondary injury of tissue, said secondary injury being induced by primary injury of mainly surrounding tissue and being the result of a traumatic event, which method comprises the administration of a physiologically acceptable vanadium compound.

1999, December 20th. Pharmaceutical composition compromising a physiologically acceptable vanadium compounds, salts and complex and at least a component selected from Na/H exchange inhibitors, cyclo-oxygenase inhibitors and caspase inhibitors.
Pharmaceutical composition for the prophylactic treatment of secondary injury of tissue, said secondary injury being induced by primary injury of mainly surrounding tissue and being the result of a traumatic event, wherein the pharmaceutical composition comprises a physiologically acceptable vanadium compound and a Na+/H+ exchange inhibitor as active components.

2001, November 16th. Method of treating burns.
A method of treating burns comprising administering to a mammal suffering from a burn one or more quercetin analogues in a therapeutically effective amount to prevent secondary injury of tissue that was not directly affected by the initial burn.

2002, October 1st. Method of treating or preventing apoptosis.
A method of treating or preventing apoptosis, wherein the method comprises the administration of an effective amount of a neuropharmaceutical selected from the group consisting of parasympathicolytics, parasympathicomimetics, sympathicolytics, sympathicomimetics and combinations thereof.

2002, October 17th. Method of treating or preventing neurodegenerative diseases.
A method of treating or preventing a neurodegenerative disorder in a mammal, said method comprising administering an effective amount of a physiologically acceptable vanadium compound to said mammal.

2003, January 8th. Method of treating or preventing vasculitis.
A method of treating or preventing vasculitis in a mammal, said method comprising administering an effective amount of a physiologically acceptable vanadium compound to said mammal.

2003, April 3rd. Method of treating or preventing neurodegenerative diseases by treating psychical stress.
A method of treating or preventing a neurodegenerative disorder in a mammal, said method comprising by preventing psychical stress.

2004, February 16th. Hair harvesting method and hair transplantation method combined with hair harvesting instruments, needle assembly, use of said instrument.

2005, November 22nd. Method for in vivo hair multiplication.
Described is a method for the reproduction of hair by removing hair in the anagen phase, in such a way that the hair stem cells that are responsible for hair growth are still attached to the removed hair, to bring it into contact with extracellular matrix components or substitutes thereof, and the implanting hair in the scalp. Also, the application of extracellular matrix components or their substitutes described for the reproduction of hair.

2006, November 23rd. Method for hair transplantation.
Described are methods and devices for hair transplantation, and in particular a special needle for harvesting of hair grafts, an implant needle for implanting hair grafts, as well as a method for harvesting of hair grafts, and a method for cosmetic hair transplant and its propagation.

2012, December 12th. In vivo Multiplication of Hair.
The present invention is in the field of cosmetic and aesthetic procedures, specifically in the field of hair multiplication technology. The present invention particularly relates to improved cosmetic methods for in vivo hair multiplication that can be used to overcome baldness. The cosmetic method of the present invention is particularly suitable for hair transplantation in recipient areas of a subject experiencing baldness or lack of hair, optionally due to Androgenic alopecia, burn injuries, cancer chemotherapy, or other genetic or environmental factors or scarring.

2013, February 1st. Composition and method for generating a desired cell type and/or tissue type from hair follicular stem cells.
The present invention is in the fields of tissue engineering with applications in the fields of cosmetic and aesthetic procedures and regenerative medicine.. The present invention provides an improved composition and in vitro method for generating a desired cell type and/or tissue type from at least one hair follicular stem cell.

2013, February 1st. Composition and method for preserving, transporting and storing living biological materials.
The present invention is in the field of living tissue bio-preservation technology. Specifically, the present invention provides an improved composition and a method for preserving living biological materials using the composition of the invention. The improved composition and method of the present invention are particularly efficient at minimizing loss of biological activity and physical integrity of living biological materials, especially in the context where living biomaterials are preserved at temperatures above freezing point.

Educational activities
Involvement in educating medical doctors and nurses in the field of hair- and scalp diseases, coordination and establish policies concerning educational projects and processes in the broadest sense as well as supervise and monitor educational processes and projects in the broadest sense at the following locations of Hair Science Institute:

- Maastricht, the Netherlands
- Amsterdam, the Netherlands
- London, the Netherlands
- Jakarta, Indonesia
- Cap d'Antibes, France
- Riyadh, Saudi Arabia
- Paris, France
- Dubai, United Arab Emirates
- Hong Kong, China

8.6 DANKWOORD

Na zoveel jaren is mijn proefschrift nu dan eindelijk klaar! De totstandkoming van een proefschrift is, zeker in mijn geval, een langdurig proces dat niet in mijn eentje tot stand is gekomen, maar dankzij directe en indirecte, persoonlijke en minder persoonlijke bijdragen van meerdere begeleiders, collegae, vrienden en familieleden tot dit mooie eindresultaat heeft geleid. Een aantal van jullie wil ik in het bijzonder noemen.

Every day I remind myself that my inner and outer life are based on labors of other men, living and dead, and that I must exert myself in order to give the same measure as I have received and am still receiving. – Albert Einstein

Allereerst wil ik mijn oprechte dank uitspreken aan mijn promotor, met zijn propeller, professor H.A.M. Neumann. Martino, wij kennen elkaar al meer dan 25 jaar. Onze relatie is gegroeid van een zuiver professionele relatie tot een zeer hechte vriendschap. Als wetenschappelijke vader heb jij mij behoed voor veel misstappen. Jij hebt aan de wieg gestaan van dit lange promotietraject van meer dan 20 jaar en voor de conceptuele input gezorgd.

Onze extreme verschillen in karakters, zoals ons tijdsbesef, maakt onze vriendschap zeer bijzonder. Echter, omdat onze neuzen altijd dezelfde kant op wijzen om doelen in het leven te bereiken, niet alleen professioneel, maar ook op persoonlijk gebied, doen we beiden onze bezigheden met veel enthousiasme.

Bedankt voor het jarenlang vertrouwen in mij en dat je me de kans hebt gegeven om als jouw laatste promovendus te promoveren. Ik ben je zeer dankbaar voor je persoonlijke, wetenschappelijke en vaak opbeurende bijdrage (ondanks de zweep) bij het voorttrekken van mijn promotie daar waar dat nodig was.

We hebben samen al veel leuke dingen meegemaakt, zowel professioneel als privé, en ik hoop samen met jou er nog veel mee te maken.

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Beste leden van de leescommissie, professor Errol P. Prens, professor Dirk Jan Duncker en professor Dimitrios Ioannides, ik dank u allen voor de tijd en moeite die u hebt genomen om mijn manuscript te lezen, erover na te denken en om aanwezig te zijn tijdens de verdediging. Dank ook voor uw snelle beoordeling. Uiteraard wil ik ook de overige commissieleden bedanken voor het stellen van slimme vragen tijdens de promotie.

Dank aan alle wetenschappelijke collega's en medewerkers die geholpen hebben bij het voltooien van de publicaties.

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inzichten en benadering van verschillende onderzoeken, hebben we uiteindelijk gelukkig altijd dezelfde doelstellingen, waardoor we ontzettend veel lol hebben in het doen van onderzoek en zo mooie wetenschappelijke resultaten hebben weten te bereiken. Ik hoop dat we in de toekomst nog aan vele wetenschappelijke en klinische projecten mogen samenwerken!

Beste collega-artsen evenals alle ondersteunende collegae van Hair Science Institute, dank voor jullie interesse en ondersteuning aangaande mijn promotieonderzoek. Jullie hebben de kliniek draaiende gehouden wat mij de ruimte gaf, tussen de behandelingen door, wetenschappelijk onderzoek te doen om zo nieuwe ontdekkingen te kunnen ontwikkelen.

Ook alle wetenschappelijke collegae met wie ik samen onderzoeken heb verricht: bedankt voor de gezellige en waardevolle discussies!

The wise woman is one who knows what she doesn't know. – Lao Tzu

Ieder persoon heeft altijd iemand nodig die een oogje in het zeil houdt en mij motiveert. Bij mij is dat iemand die mij nauw aan het hart ligt, maar altijd op de achtergrond blijft en zal blijven.

Life is a journey to be experienced. Not a problem to be solved. – Alan Alexander Milne (Winnie-the-Pooh)

De indirecte en directe betrokkenheid van mijn vrienden en (schoon) familieleden en het uitwisselen van levenservaringen met hen, heeft mijn leven niet alleen tot een grotere vreugde gebracht, maar heeft minstens evenveel bijgedragen aan het voltooien van dit proefschrift. Daarbij wil ik toch twee vrienden in het bijzonder noemen.

Jan, jij en ik kennen en begrijpen elkaar al vanaf de middelbare school, waar wij veel met elkaar deelden, zelfs kauwgom. Alhoewel onze interesses sterk van elkaar verschillen, willen we allebei hetzelfde in het leven bereiken. Ik ben blij jou weer aan mijn zijde te hebben, zowel privé als beroepsmatig. Na al deze jaren ben ik nog altijd onder de indruk van jouw loyaliteit en gedrevenheid. Dat wij nog lange tijd onze vriendschap zullen koesteren.

Sen, onze vriendschap is begonnen aan het begin van onze studie geneeskunde aan de Erasmus Universiteit in Rotterdam. Wij hebben een leuke studententijd gehad, samen genieten van lekker Indisch eten (toen nog klaargemaakt door onze lieve moeders), sporten & natuurlijk uitgaan, dat regelmatig werd beëindigd met een nachtelijk bezoek aan een eettent in de Witte de Withstraat (wat soms resulteerde in vreemde ras-gerelateerde laboratoriumuitslagen).

Maar er moest natuurlijk ook gestudeerd worden, alhoewel ik vaak werd weggestuurd uit de bieb door onze vriendengroep omdat ik te snel klaar was met studeren en dit demotiverend werkte. Laat nu een aantal personen van deze vriendengroep veel eerder zijn gepromoveerd en sommigen zelfs hoogleraar geworden zijn. Eén van deze vrienden heeft nu ook zitting in één van mijn commissies. En ik hobbel er nu achteraan...

Vanaf het einde van onze studententijd zijn ook onze wederhelften, Yvonne & Sheila, vriendinnen van elkaar. Zij zorgen sindsdien er samen voor dat al het lief en leed van inmiddels ook onze gezinnen met elkaar worden gedeeld. Het is leuk om te zien hoeveel gelijkenis de levensloop van onze gezinnen vertoont: niet alles in het leven is rozengeur en maneschijn. Hopelijk zullen we nog jarenlang samen van onze vriendschap genieten.

Met de (schoon) familieleden, zowel in Nederland als ook in Indonesië was het ook altijd genieten tijdens de vele gezellige bijeenkomsten.

Dank jullie wel allemaal!!

He who does not trust enough, will never be trusted. - Lao Tzu

Alhoewel zij helaas beiden niet meer in ons midden zijn, zou ik toch mijn schoonouders, Rob en Betsy Njo, willen bedanken voor het vertrouwen dat zij in mij hadden als schoonzoon vanaf het moment dat ik met Sheila omging, nu meer dan 30 jaar geleden. Mijn schoonouders en ouders waren in Jakarta lid van dezelfde studentenvereniging, en wellicht heeft dat hen enigszins gerust gesteld (of juist niet). Als ouders wil je het allerbeste voor je kind, maar met name voor een vader is het moeilijk om een dochter los te laten.

Ook mijn zwagers, Arby en Brian, die altijd bereid zijn om te helpen, wil ik bedanken; niet op zijn minst ook voor de zinvolle en minder zinvolle discussies.

Silence is a source of great strength. – Lao Tzu

Mijn ouders, Lena en Tjeng Hoei Gho. Mammie en Daddy, heel veel dank voor de offers die jullie hebben gebracht, zoals het emigreren van Indonesië naar Nederland. Hier heb ik mij kunnen ontwikkelen en vormen tot wie ik nu ben; jullie zijn mede verantwoordelijk voor wie ik ben geworden en wat ik tot nu toe heb kunnen bereiken. Samen hebben jullie mij geleerd om te genieten van wat je hebt, het leven te accepteren zoals deze je is gegeven, geluk te waarderen, en niet te kijken naar wat je mist. Dit is wellicht één van de redenen waarom het schrijven van dit proefschrift zo lang heeft geduurd, maar tevens ook een reden en motivatie om dit proefschrift tot een goed einde te volbrengen.

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In het bijzonder wil ik stilstaan bij mijn vader, een man van weinig woorden. Het doet pijn dat hij mijn promotie net niet meer heeft kunnen meemaken, maar één van zijn laatste wensen komt wel in vervulling. Bij ons laatste samenzijn zei hij: "Je proefschrift is af en je heb in ieder geval de datum vastgelegd." Zijn levenswijze en visie betreffende de essentie van het leven hebben mij niet alleen in gelukkige tijden, maar ook in mindere perioden geholpen om deze vrij ongeschonden door te komen.

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Niet voor niets gaat Ben mij wetenschappelijk bijstaan als één van mijn paranimfen: hij is een wetenschapper in hart en nieren.

"How do you spell love? You don't spell it.... You feel it." - Alan Alexander Milne (Winnie-the-Pooh)

Volgens traditie komen in het dankwoord de belangrijkste personen als laatste… en ook hier is dat niet anders. Naast al het reilen en zeilen rondom Hair Science Institute, zowel in Nederland als ook in het buitenland, ging veel van onze kostbare tijd in mijn promotie zitten.

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Ik hou heel veel van jullie drie!

Coen.

FOLLICULAR STEM CELLS AND THEIR POTENTIAL APPLICATIONS

During the last decade, the interest in autologous stem cells has increased considerably, especially regarding the development of individualized therapies. If follicular stem cells from plucked hair could be used for stem cell therapy, their practical utilization for autologous stem cell therapy would increase immensely.

In this thesis we revealed that follicular stem cells are located in the bulge area of the hair follicle and absent in or near the dermal papilla. Furthermore, follicular stem cells can be obtained from cells derived from plucked hair and are retained in cultures made from these cells.

Hair loss is a common problem with different underlying causes. Hair transplantation is the only method to restore hair permanently. In all techniques of hair transplantation, follicular stem cells are important because they regenerate new hair follicles. We demonstrated that extracted partial longitudinal follicular units (PL-FU's) preserved in a solution containing Bis(maltolato)oxovanadium (BMOV) and transplanted to the recipient area can be used as complete follicular units to regenerate completely differentiated hair growth with the same characteristics as in the donor area. On the basis of this, the partial longitudinal follicle unit transplantation (PL-FUT) was developed and has shown to be an effective technique for androgenic and cicatricial alopecia with a high patients satisfaction, minimal side effects, natural result and preservation of the donor area. This technique is also called hair stem cell transplantation because only a portion of the total amount of donor hair stem cells is transplanted.

The same human hair follicle bulge-derived stem cells can also be cultivated easily, expanded efficiently and kept frozen until needed, and therefore they are suitable as a source for future (neural) regenerative therapies.