



A Presentation of
Ongoing Clinical Study Data
for the
REVÅGE670™ Laser System



100 Bayview Circle, Suite 2200 • Newport Beach, CA 92660
Phone: 866.982.7472 • Email: info@apriascience.com



Introduction

The Revåge670 Laser System began life in Sweden more than a decade ago. The inventors of the product developed the system in response to a growing scientific community belief that Low-Level Laser Therapy (LLLT) was a viable therapeutic option for wide array of human skin disorders. Researchers in Sweden, Russia, Japan and Hungary had all demonstrated that this emerging technology held a great deal of promise.

The Revåge670 Laser System entered the United States as a cosmetic product for application as therapy for improving the quality of hair, which is subject to great variability in both the scope and range of interpretation.

A U.S. patent, entitled "Method and Device for Stimulating the Activity of Hair Follicles", was issued for this technology in December of 2003 (# 6,666,878). Since that date, the proponents of the technology have endeavored to demonstrate, through empiric data gathering, that the Revåge670 Laser System is indeed an efficacious device whose scientific validation is closer than previously believed.

This presentation seeks to provide the photomedicine specialist with an overview of the ongoing research sponsored by Apira.

Table of Contents

	Page
Research Projects to Date	2
Interim Clinical Data – Overview Report	3
Hair Pigmentation: A Research Update	9
The Revåge670 Technology	14
Research Opportunities with Apira	14



Research Projects to Date

1. 2005 to present: Beta site studies have been conducted in the USA, in the private practice offices of plastic surgeons, dermatologists and facial plastic surgeons.
2. A comparative study of LLLT (the Revage670) versus LED therapy for the treatment of androgenetic alopecia was completed in 2007. The format of the study was a randomized, double blind, multi-center, comparative trial. The study was conducted at three locations, with physician investigators owned by a group of Hair Transplant Specialists. It is anticipated that the results of this study will be submitted for publication in the third quarter of 2008.
3. A human tissue biopsy study was begun in 2007 and currently continues. This study is being conducted by a dermatologist and plastic surgeon in cooperation with the Wellman Laboratories for Photomedicine. Interim data from this study is offered in this presentation.
4. A randomized, double blind, placebo-controlled, single center trial to investigate the potential benefit of the Revage670 Laser System for the prevention of hair loss during chemotherapy, will begin in the last quarter of 2008.
5. A single site, open trial is scheduled to begin in April of 2008, with the Revage670 Laser System to investigate the potential benefit of the LLLT therapy for eyebrow restoration secondary to alopecia totalis.

Note: All trials, excluding the beta site work, are conducted under IRB approval and follow strict GCP guidelines.



Interim Study Data – Summary Report

1. Comparative Trial Conducted in 2007 by Hair Transplant Physicians – Research Project Number 2

Title

A Comparative Study of LLLT versus LED therapy for the treatment of androgenetic alopecia in adult males.

Objective

To evaluate the difference in therapy between an LLLT device, the Revâge670 Laser System operating at 660 nanometers, and a Revâge670 with the laser diodes removed and replaced with red light super luminescent diodes.

Sites

Physician managed offices located in Orlando, Cleveland and Houston that specialize in Hair Transplant Procedures.

Materials and Methods

The Revâge670 Laser Systems were supplied by the manufacturer in two configurations. One fully operational system with laser diodes and a one system configured with only LEDs was installed at each location. The study was projected for a 24-week duration. An internal clinical evaluation committee reviewed the proposed protocol and agreed to conduct the trial according to Good Clinical Practices. Thirty-six subjects were selected based upon the following inclusion criteria:

- Males ages 18 - 48
- Norwood Hamilton class IIa – V and Fitzpatrick Phototypes I – IV
- Absence of active infections or other hair restoration medications
- Permit shaving and tattooing of treatment areas
- Permit photography and publication of photographs
- Agree to return for hair counting sessions
- No compensation was offered, only the no-cost therapy
- Informed Consent document was signed

Treatment Protocol

On the first post-initial evaluation session, the area to be irradiated was shaved and tattooed according to a standard established by the protocol. The Principal Investigator and his associates had conducted numerous trials for major manufacturers of pharmaceuticals, as well as devices used in the hair restoration field of medicine.

Photographs were taken and the first treatment session was performed. Treatment sessions were administered three times per week for one month and two times per week for five months. Each session was 20 minutes in duration.

Adverse Events

At each treatment session, subjects were questioned about any complaints or untoward effects. An MHR physician also performed an examination of the treatment area to determine if there were any visible sign of erythema, excoriations, dryness, edema or paresthesia. There were none reported.

Summary of Results

Hair counting was performed prior to the first treatment session and immediately thereafter. Immediately thereafter is defined as within seven days of the last treatment. All hair counting was performed by a non-participating physician from digital photographs taken at the investigational sites. In the arm treated with the Revâge670 Laser System, there was a 68.75% increase in terminal hair counts and 31.25% registering no change. No subjects demonstrated a loss of terminal hairs.

In the arm treated with the super luminescent diodes there was a 38.5% increase in terminal hair counts, 30.75% registering no growth and 30.75% demonstrating a loss of incremental terminal hairs.

The data from this clinical trial suggests that the Revâge670 Laser System is 44% better than the LED system. What is most significant to the sponsor is the hair loss that continued with the LED was not observed with the laser.



2. A Basic Science Investigation Designed to Elucidate the Mechanism of Action, at the Cellular Level, for Red Laser Light Therapy and Hair Re-Growth

Title

The physiologic effects of Low Level Laser Therapy (LLLT) on human follicular cells and surrounding dermal tissue structures.

Objective

This study on LLLT aims to define the safety and physiologic effects that occur when the human hair follicle and surrounding tissue structures are exposed to this type of radiation. Thus far, all reports on the efficacy of laser light in this area, have been unsupported by basic science data. The data available has been the result of testing murine models, not human. To properly identify the effects in human subjects, exposure to LLLT is not sufficient. The analysis of the non-radiated and radiated tissues is required to elucidate the tissue response and efficacy of the laser therapy. The theory that is widely accepted is that the mitochondria are the powerhouse of the stem cells that cause hair growth. The LLLT “turns on” the nutrient pump process that energizes the mitochondria, which leads to an increase in ATP and subsequent reversal of hair follicles from the dormant stage of growth called telogen, to the active growth stage called anagen.

Sites

This is a single site study. The length of time required to complete the study, under this protocol, inclusive of all subject therapy and tissue harvesting will not exceed 16 weeks. All subjects, of which there will be five in total, will be recruited by the Principal Investigator from his pool of medical colleagues and associates. No advertising of any type will be required to recruit these five subjects.

Material and Methods

Male and female subjects experiencing any type of hair loss, thinning hair, or androgenetic alopecia. The total number of subjects being recruited is five; three female and two male. Age range is 19 - 59 years. Apparent good health. Each participant will be subject to a brief physical examination and the taking of a medical history including information on current treatment and prescription drugs. No blood work will be required unless the cause of the hair loss is undetermined. If blood work is

required, the analysis will focus on thyroid, estrogen and testosterone serum levels only. No previous involvement in other hair studies. No use of any hair growth agent within the last six months. No evidence of any current viral, fungal or bacterial infection.

Treatment Protocol

The LLLT device that will be used is called the REVÅGE670™. This is a low-level diode laser system, operating at 670 nanometers, that contains thirty 4-milliwatt diode lasers affixed in a rotating helmet. The subject's head is positioned within the helmet, which is similar to a salon hair dryer, until a sensor triggers the start of therapy. There is no contact between the subject and the device; only the laser light reaches the subject's scalp. Treatment lasts for approximately 15 minutes, but is dependent on skin type classifications. The laser automatically shuts off after the treatment session is complete. There is no pre or post treatment care required, only that subject hair must be clean and not contain spray or gel fixative agents. No safety eyewear is required during the treatment session.

After recruitment is completed, the five subjects will be evaluated to determine Fitzpatrick skin type, which is required for prescribing the appropriate length of each laser treatment session. Each subject will receive two treatments per week for six weeks and one treatment per week for six weeks, totaling 18 laser treatments. The area of treatment will be the vertex of the scalp. The initial visit will include the harvesting of pre-treatment tissue samples. After the 18th treatment, tissue samples will be harvested again from the designated area of laser treatment. It is proposed that this algorithm will cause the physiologic changes to occur at the cellular level, which is theorized by experts in the field of Photomedicine and LLLT.

There are no validated questionnaires available for querying subjects about hair loss treatments and subsequent outcomes. Therefore, no questionnaires or subject diaries will be employed in this study.

Prior to the first laser treatment session, but on the same day, a 3mm punch biopsy will be harvested from the area designated as the treatment area for the laser. More than one

punch may be harvested. This determination will be made by the physician at the time of harvesting tissue. The procedure for harvesting the tissue is as follows:

- A sterile field will be prepared
- 1% lidocaine with epinephrine, (1-100,000) will be injected to provide anesthesia
- A single use, 3mm punch will be used to harvest the tissue sample
- A 6/0 dermal, polypropylene suture will be used to make a primary closure of the site
- The suture will be removed between 7 and 10 days post-closure
- The tissue sample will be labeled with a number ONLY. A reference will be maintained in the subject's medical record identifying the tissue sample and the subject.
- The tissue sample will be immersed in a preservative container and shipped to a research laboratory where the tissue analysis will be performed under a different IRB approval. The laboratory is Wellman Center of Photomedicine. After analysis is completed by Wellman, the tissue samples will be incinerated.
- A statement of findings from the Wellman Center researchers will be submitted to the IRB upon completion of the study in its entirety.

Adverse Events

There are no known risks or discomforts associated with the laser treatments administered with the Revage670. Although it is not anticipated, there is a very slight risk of bruising, scarring and infection from the tissue harvesting and blood drawing procedures. All possible precautions will be taken to prevent these unwanted effects. No other risks are anticipated. All subjects will be monitored for changes in health status or for indications of any form of infection in the areas where tissue was harvested. The monitoring will be performed, on each visit, by the physician investigator and his staff.

Summary of Results

There were eight before and after biopsies taken from four subjects to compare. There were four outcome measures: (a) number of hairs, (b) presence of anagen hairs, (c) number of hairs containing Melanin, (d) presence of Ki67, a marker of proliferating cells in the hair follicles. ALL FOUR subjects showed improvement in at least one of these measures in at least one pair of biopsies. Subject 5 had the most impressive overall improvement.

Additional tissue samples are being harvested at present to continue the analysis. There has been a learning curve to overcome in the process of performing biopsies. It has been learned the clinician must harvest tissue samples that are as close to the pre-treatment location, in the post-treatment location. The investigators had not adequately considered the essential importance of exact location at the outset of this study. It is acknowledged that this specific research is extremely rare and requires a refinement of the techniques employed to properly harvest, prepare tissue samples in the lab and evaluate the data acquired. An increase in irradiation events is also part of the new protocol going from 16 weeks to 22 weeks.

Each of the four subjects demonstrated an increase of melanogenesis. Based upon work published by Tobin, Hordinsky and Bernardz in the J. Investig Dermatolo Symp Proc 10:275 – 279, 2005 (attached) melanogenesis in the hair shaft, bulge and outer root sheath occurs only in Anagen I – IV. Such published research supports the theory that if red laser light causes an increase of melanogenesis, it must be stimulating the production of Anagen hairs, which will lead to more terminal hairs.

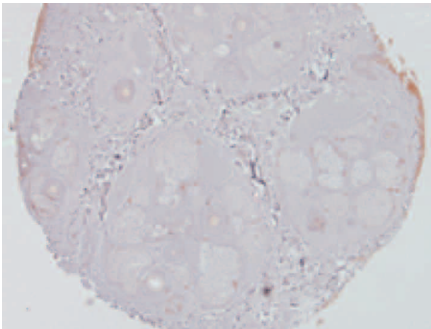
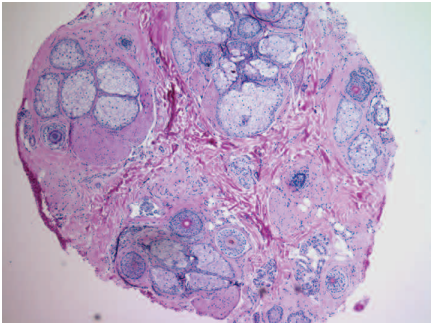
The following are histology examples which demonstrate increases in melanogenesis and anagen hairs. The A and B are designations for pre-irradiation samples and C and D are for post-irradiation samples.

- 1 A & 1 C – Increases in melanized hairs and more Ki67 positive cells in follicles
- 3 A & 3 C – Increases in melanized hairs and more Ki67 positive cells in follicles and more anagen hairs
- 4 A & 4 C – Increases in anagen hairs
- 4 B & 4 D – Increases in melanized hairs and more Ki67 positive cells in follicles
- 5 A & 5 C – Increases in anagen hairs, increases in melanized hairs and more Ki67 positive cells in follicles
- 5 B & 5 D – Increases in anagen hairs, increases in melanized hairs and more Ki67 positive cells in follicles

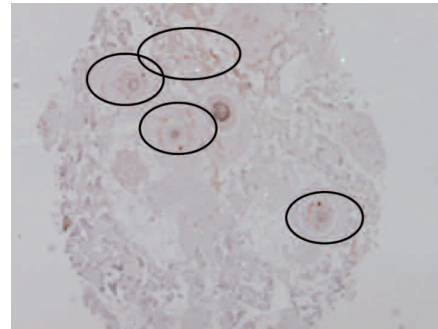
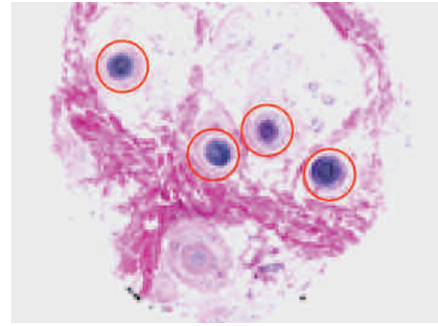
Before

After

1A

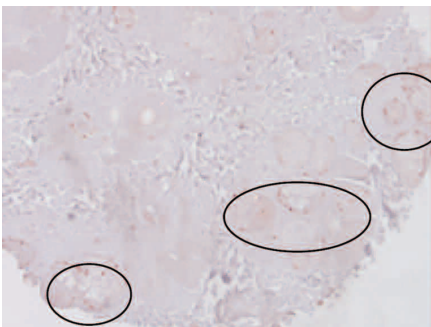
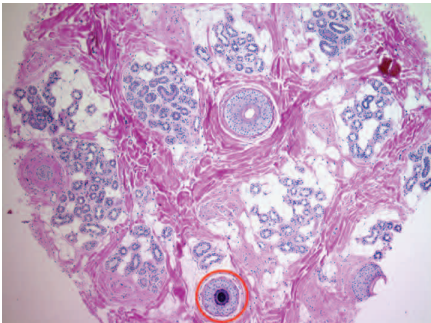


1C

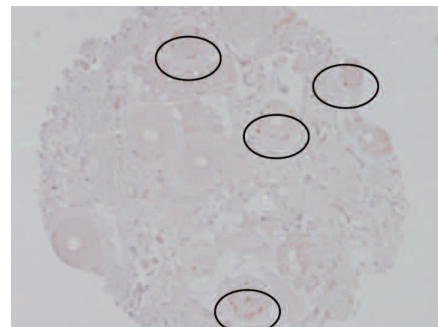
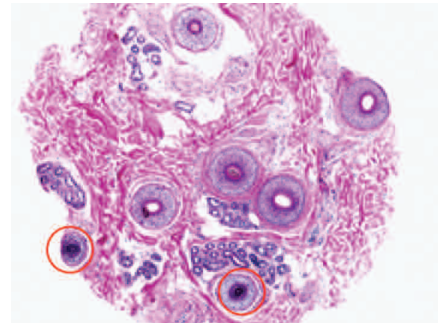


No sign of more hairs or more anagen hairs in either region. However, comparing 1C with 1A we see more melanized hairs and more Ki67 positive cells in follicles (circled, more cellular proliferation). Therefore the LLLT appears to have had some early beneficial effect in region of 1A and 1C.

3A



3C

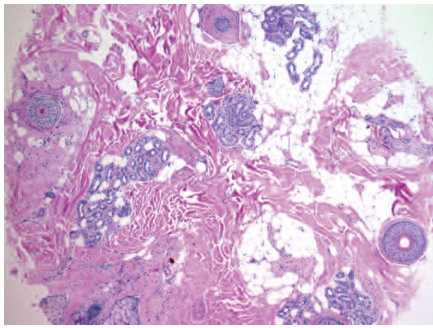


3C had more hairs and more anagen hairs compared to 3A. There was also more melanized hairs and more Ki67 staining. Therefore the LLLT appears to have had some overall beneficial effect in region of 3A and 3C.

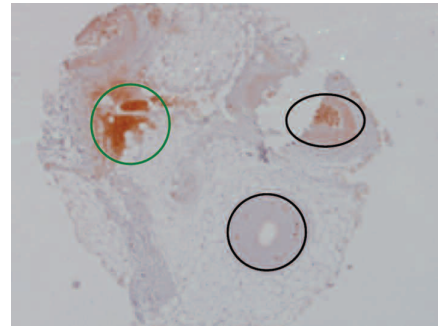
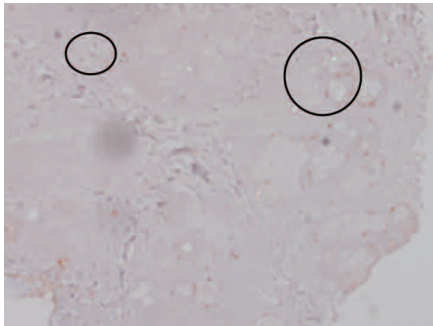
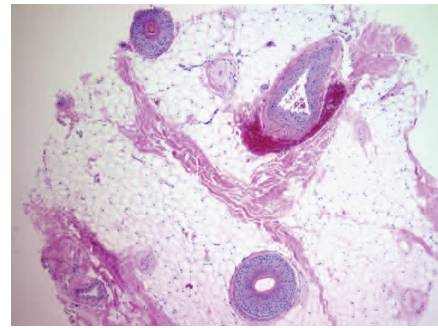
Before

After

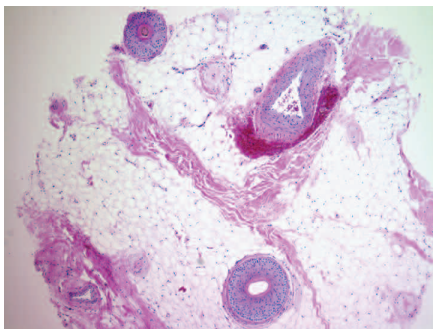
4A



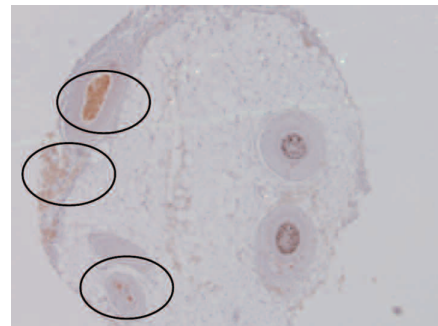
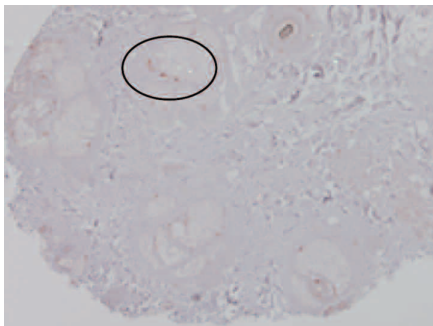
4C



4B



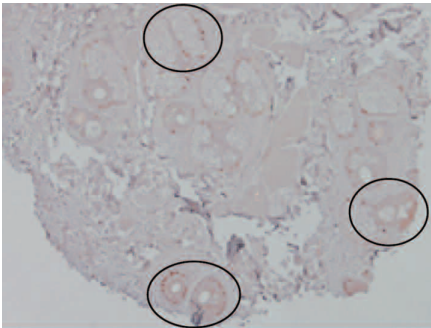
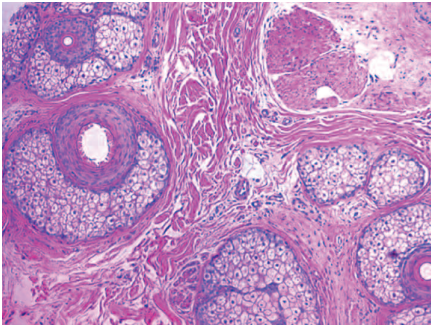
4D



Increase in anagen hair in 4C vs 4A no increase in number. Increase in melanin in 4D vs 4B. Increase in Ki67 staining in 4D vs 4B (green circle is an IHC artifact). Again a moderate overall benefit.

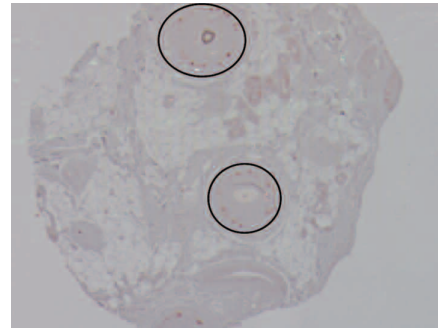
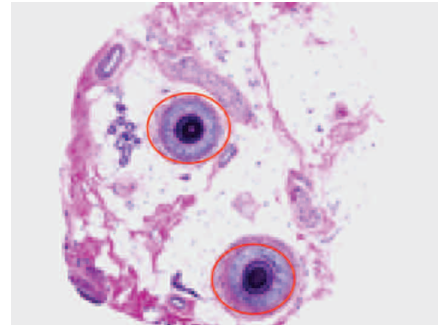
Before

5A

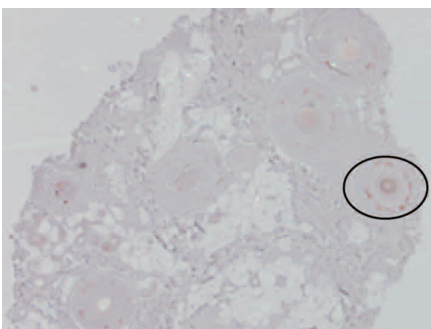
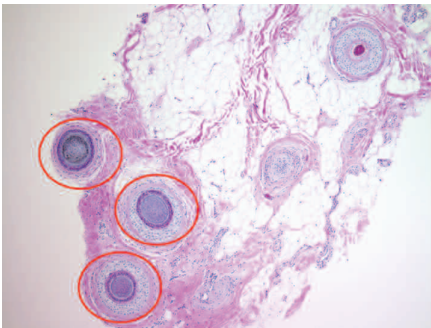


After

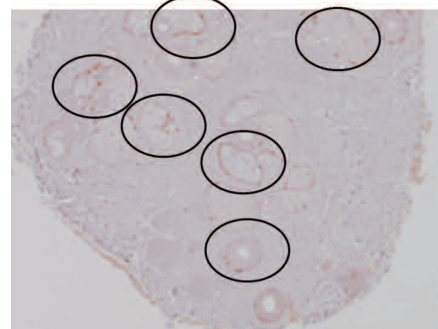
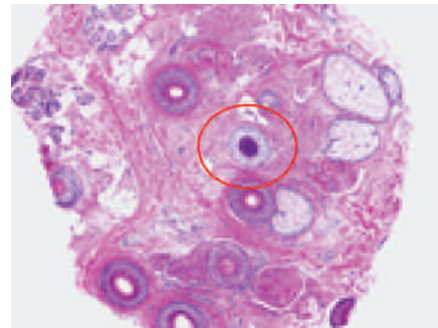
5C



5B



5D



More hairs and more anagen hairs in both sections. Increase in melanized hairs in 5C vs 5A and large increase in Ki67 staining in 5D vs 5B. Subject 5 was the best overall result.

Hair Pigmentation: A Research Update

Desmond J. Tobin,* Maria Hordinsky,[†] and Bruno A. Bernardz[‡]

*Department of Biomedical Sciences, University of Bradford, Bradford, West Yorkshire, UK; [†]Department of Dermatology, University of Minnesota, Minneapolis, Minnesota, USA; [‡]L'Oreal Recherche, Clichy cedex, France

Hair is a uniquely mammalian trait with important functions, most easily appreciated in furred mammals. Our skin and hair color contribute very significantly to our overall visual appearance by highlighting striking variations between human subgroups. Although melanins, hemoglobins, and carotenoids define the color perceived at the skin surface, our hair color relies only on the presence or absence of different melanins. The hair shaft's physical aspects provide only minor color modification. Various selective evolutionary pressures have determined that within the context of our specific ethnic backgrounds a bewildering array of natural shades are seen; ranging from yellows, reds, and browns to black and that harbinger of lost youth, gray/white hair. Skin/hair follicle melanins are formed in cytoplasmic organelles called melano-somes produced by neural crest-derived pigment cells called melano-cytes and are the product of a complex, phylogenetically ancient, biochemical pathway called melanogenesis. The following provides a review of research presented at the 4th *Intercontinental Meeting of Hair Research Societies 2004* and so is not intended to represent a fully comprehensive overview of the subject—for that readers are directed to key references.

Key words: hair follicle/melanocyte/pro-opiomelanocortin/hair cycle/nitric oxide synthase
J Investig Dermatol Symp Proc 10:275–279, 2005

Development of the Hair Follicle Pigmentary Unit

By 7 wk estimated gestational age (EGA) melanocytes are already present in the human epidermis and remain there until hair morphogenesis begins, approximately 2 wk later (cf. Tobin and Paus, 2001). With the onset of hair follicle morphogenesis, some melanoblasts leave the epidermis and distribute randomly as dopa-positive and dopa-negative cells in the forming hair follicle and occasionally in the sebaceous glands. Melanogenic melanocytes can be detected in all stages of human hair morphogenesis from the hair germ stage onwards, usually confined to the peripheral regions of the hair follicle. Once the hair fiber has formed, melanocytes are noted in large numbers near the basal lamina around the apex of the follicular papilla. Although melanocyte mitosis is observed in the human epidermis at 14 wk EGA, mitosis of pigment cells is rarely observed in the hair follicles themselves.

Early work by Nishikawa *et al* (1991) and Yoshida *et al* (1996) demonstrated an important role for c-kit in melanocyte development. C-kit is required for melanocyte migration in the dermis (along with endothelin 3), but not just before their entry into the epidermis. C-kit signaling is again needed for their proliferation upon entering the epidermis (with the endothelin receptor B but not endothelin 3), but this is followed by a c-kit-independent stage upon entry into the developing hair follicles. C-kit is thereafter required for melanocyte activation during reconstruction of the hair follicle pigmentary unit life.

More recently it has been shown that melanoblasts expressing c-Kit migrate into the stem cell factor (SCF)-supplying hair follicle epithelium and that differentiated c-Kit-positive melanocytes target the bulb when "SCF positive" (Peters *et al*, 2002). C-Kit-negative melanoblasts invade the outer root sheath and bulge in the fully developed murine hair follicle. Postnatally, c-Kit is required during the hair growth cycle for activation of melanocyte, although the stem cell compartment appears to exhibit SCF/c-kit independence. The cutaneous expression of SCF (produced by fibroblasts, keratinocytes, and endothelial cells) and of its receptor c-Kit has recently been re-evaluated (Peters *et al*, 2003). Mast cells and melanocytes were thought to be the only cutaneous cell types that express c-Kit and so to respond to SCF. Recently, Peters and colleagues reported the rather unexpected and exciting finding that Kit expression in murine anagen hair follicles is not restricted to the pigmentary unit, but is also present in desmoplakin⁺/Trp-1-negative cells (identified as keratinocytes). Indeed, these c-kit-positive cells were also found in melanocyte-deficient hair follicles *Kit^{Sl}/Kit^{Sl-d}* mice, and in Kit-neutralizing antibody-treated C57BL/6 mice. Moreover, *Kit^{Sl}/Kit^{Sl-d}* mice unable to respond to SCF stimulation showed a significant retardation of anagen development compared with their wild-type littermates. Thus, expression of Kit in the most rapidly proliferating compartment of the hair follicle epithelium suggests intriguing, as yet unexplored, functions of Kit signaling in epithelial cell biology.

There is some evidence that fibroblast-like cells of the follicular dermal papilla may secrete more SCF than dermal fibroblasts and that follicular dermal papilla cells from beard hair follicles may secrete more SCF than androgen-insensitive scalp hair follicles (Hibberts *et al*, 1996). Two different splice variants for SCF encode for either a soluble or a membrane-bound form. Dr Tayyeb Vafae (Dr Valarie Randall's Group) reported at this workshop that human hair follicles, especially their lower part, express the genes

Abbreviations: BMP, bone morphogenic proteins; CRH, corticotrophin-releasing hormone; MC1-R, melanocortin 1-receptor; MSH, melanocyte stimulating hormone; NO, nitric oxide; NOS, NO synthase; POMC, pro-opiomelanocortin; SCF, stem cell factor



for both the soluble and membrane-bound forms of SCF. These workers did not comment on the expression of the SCF isoforms at the protein level, and so it is not yet clear how this observation may affect the biology of the hair follicle including pigmentation. Other researchers have shown that soluble and membrane-bound SCF may even have opposite functional effects.

Distribution and Antigen Expression of Hair Follicle Melanocytes

Although follicular melanocytes are derived from epidermal melanocytes during hair follicle morphogenesis, these pigment cell sub-populations diverge in many important ways as they distribute to their respective distinct compartments. Thus the follicular-melanin unit resides in the “immune privileged” proximal hair bulb (cf. Tobin and Paus, 2001) and consists of one melanocyte for every five keratinocytes in the hair bulb as a whole; the ratio is 1:1 in the basal epithelial layer next to the dermal papilla. Hair bulb melanotic melanocytes differ from epidermal melanocytes in that they are larger, with longer and more extensive dendrites, containing more developed Golgi and rough endoplasmic reticulum, and produce 2–4-fold larger melanosomes. Moreover, whereas melanin degrades almost completely in the differentiating layers of the epidermis, eumelanin granules transferred into hair cortical keratinocytes remain minimally digested.

Melanocytes in the fully developed anagen follicle can be assigned to five distinct anatomic compartments. Dopapositive melanotic melanocytes are readily detectable in the outer root sheath of the infundibulum, basal layer of sebaceous gland, and around the upper dermal papilla. Dopa-negative amelanotic melanocytes are detectable in the mid-to-lower outer root sheath, and the amelanotic dopa-negative melanocytes are distributed in the periphery of the bulb and most proximal matrix. The melanocyte stem in murine hair follicles has been localized to the bulge region (Nishimura *et al*, 2002). Immature melanocytes can be clearly demonstrated in the adult hair follicle (Tobin and Paus, 2001; Slominski *et al*, 2004b). Immunostaining with NKI-beteb, a monoclonal antibody that detects the (pre)melanosome glycoprotein gp100, detects all dopapositive cells and also highlights some dopa-negative melanocytes of the mid-outer root sheath. Although the dopaoxidase activity of tyrosinase is not detectable in amelanotic hair follicle melanocytes, the protein itself may be detected in some of these cells. Similarly, whereas Kit and Bcl-2 reactive melanocytes are present in this hair follicle compartment, these amelanotic melanocytes do not express the melanogenic enzymes TRP-1 and TRP-2 (also called dopachrome tautomerase). The hair bulb, however, is the only site of pigment production for the hair shaft, and contains both highly melanogenic melanocytes and a minor subpopulation of poorly differentiated, NKI-beteb⁺, pigment cells (cf. Tobin and Paus, 2001; Slominski *et al*, 2004a, b). It has been speculated that amelanotic hair bulb melanocytes may represent a pool of “transient” melanocytes that migrate from precursor melanocytes stores in the upper outer root sheath. The restriction of melanogenically active

melanocytes to the upper hair matrix of the anagen hair follicle, just below pre-cortical keratinocytes, correlates with the observation that melanin is transferred during anagen to the hair shaft cortex, less so to the medulla and rarely to the hair cuticle.

Hair Follicle Melanogenesis Is Coupled to the Hair Growth Cycle

Activity of the hair bulb melanocyte is under cyclical control and melanogenesis is tightly coupled to the hair growth cycle (cf. Slominski *et al*, 2004a, b), in contrast to epidermal melanogenesis, which appears to be continuous. Hair grows cyclically through a finite period of hair shaft formation (anagen ~ 3–5 y in human scalp), a brief regression phase resulting in the apoptosis-driven resorption of up to 70% of the hair follicle (catagen ~ 2 wk in human scalp), and a relatively quiescent period (telogen ~ 3 mo in human scalp). Even before catagen-associated structural changes, towards the end of anagen VI, the earliest signs of imminent hair follicle regression become apparent, e.g., retraction of melanocyte dendrites and attenuation of melanogenesis. Keratinocyte proliferation, however, continues for some time and the most proximal telogen hair shaft remains unpigmented. The melanogenically active melanocytes of the anagen phase are no longer detectable during catagen. The hair bulb melanocyte system has long been viewed as self-perpetuating, whereby melanocytes involved in the pigmentation of one hair generation are also involved in the pigmentation of the next. But melanocytes would need to survive/avoid the extensive apoptosis-driven regression of the hair bulb. Recently we have found that many highly melanotic hair bulb melanocytes do not survive catagen (cf. Tobin and Paus, 2001; Slominski *et al*, 2004a, b). Thus, it now appears that “re-differentiating” melanocytes in early anagen are more likely to correspond to newly recruited immature melanocytes derived from a melanocyte reservoir or stem cell pool (Tobin *et al*, 1999; Nishimura *et al*, 2002) located in the upper, “permanent,” outer root sheath (Commo and Bernard, 2000). This view is supported by the observation that immature melanocytes are located very close to the secondary epithelial germ of the telogen club where they are commonly small, have high nuclear to cytoplasmic ratios, and inactive cytoplasm with very few organelles.

Melanin synthesis is not detected by histologic or histochemical examination in telogen hair follicles, but very low tyrosine hydroxylase activity of tyrosinase can be detected; this disappears on days 1 and 2 after anagen induction in mice (cf. Slominski *et al*, 2004a, b). Undifferentiated melanocytes/ melanoblasts of the telogen germ are stimulated at the start of anagen and respond by increasing their cell volume. This anagen-associated signal predates the melanogenic stimulus delivered during anagen III and is followed by active melanogenesis and subsequent transfer of mature melanosomes into pre-cortical keratinocytes. Melanocytes in the S-phase of the cell cycle occur as early as anagen II and significant proliferation is clearly apparent by anagen III (Commo and Bernard, 2000). Mitosis is also observed in melanogenically active cells, indicating that melanocyte differentiation does not

preclude mitotic activity.

In the murine telogen hair follicle some melanocytes/melanoblasts from the secondary germ are immunohistochemically positive for TRP-2; of these, a subpopulation also expresses c-Kit. All telogen melanocytes are mitotically quiescent as assessed by Ki67 immunostaining. During anagen I, tyrosinase mRNA and tyrosinase protein become detectable, but barely [and melanin] TRP-1, and tyrosine hydroxylase and dopa oxidase activities of tyrosinase remain undetectable at the biochemical level. TRP-2-positive melanocyte precursor cells proliferate extensively at the onset of follicle development and thereafter assume a more differentiated status that is associated with the onset of TRP-1 expression and with the reduction of their proliferation (Botchkareva *et al*, 2003). At the same time, melanocytes residing at the site of the presumptive stem cell reservoir in the infundibulum remain TRP-1 negative, whereas some cells expressing TRP-1 or TRP-2 together with c-Kit may show proliferative activity (Botchkareva *et al*, 2001).

During murine anagen II tyrosine hydroxylase and dopa oxidase activities of tyrosinase, tyrosinase mRNA, protein, TRP-1, and melanin are readily detectable, to increasing rapidly during anagen IIIc (day 5 after anagen induction) and reaching their highest levels during anagen V and early anagen VI (days 8–12) (cf. Slominski *et al*, 2004a, b). By anagen IV, when the hair pigmentary unit becomes fully functional with respect to melanin synthesis, melanocytes distribute into discrete locations throughout the hair follicle. In murine hair follicles, melanocytes localized to the HF bulge (site of presumptive reservoir) express only TRP-2, lacking TRP-1, c-Kit, and Ki67 immunoreactivities. Melanocytes located in the elongating outer root sheath are TRP-2+ and c-Kit+ and in some cases are also positive for the proliferation marker Ki67, but express little TRP-1 and no tyrosinase (Botchkareva *et al*, 2001). Only melanocytes distributing to the hair follicle melanogenic zone, i.e., the matrix above the dermal papilla, express TRP-1, TRP-2 and tyrosinase, c-Kit, and also Ki67 in the majority of cells. Both activity and concentration of tyrosinase remain steady during mid-to-late anagen VI (days 12–17), and decrease rapidly during the anagen VI/catagen transition phase to become undetectable or very low in catagen. Melanocytes from the fully developed anagen VI murine hair follicles express TRP-2 only when located in the hair bulge, TRP-2 and c-Kit only when located in the outer root sheath, and all three proteins, together with c-Kit, when located in the hair bulb matrix. Melanocyte proliferation, however, ceases by this sub-stage of anagen. The physiologic decrease in follicular melanogenesis at the end of anagen may reflect an exhaustion of an active signaling system that stimulates melanogenesis (late anagen VI), and/or production of inhibitors of melanocyte activity. Active depletion of differentiated hair bulb melanocytes via apoptosis also contributes to termination of melanogenesis during late anagen/catagen transition phase (cf. Tobin and Paus, 2001; Slominski *et al*, 2004b).

Null mutations and deletions at the loci for tyrosinase and TRP-1 phenotypically affect coat color because of absence of enzyme activity or because of intracellular mislocalization. Mutations (although not null mutations) have been described at the TRP-2/Dct locus, which also lead to a pigmentation phenotype. A forced TRP-

2 null mutation has been reported to result in a diluted coat color phenotype with reduced melanin content. Although tyrosinase is absolutely required for melanogenesis, other melanosomal proteins, such as TRP-1, TRP-2, and gp100, play important roles in regulating mammalian pigmentation. For example, whereas TRP-1 can oxidize 5,6-dihydroxyindole 2-carboxylic acid (DHICA) in mice but not humans, TRP-1 may play an important role in stabilizing tyrosinase in both species.

During this workshop Dr Stéphane Commo (Dr Bruno Bernard's Group) reported the rather unexpected finding that, in contrast to mice, melanogenic melanocytes of the human anagen hair bulb (at least of those hair follicles producing eumelanin, i.e. brown and black hair fibers, irrespective of ethnic origin) do not express TRP-2 at the protein level. Moreover, whereas only a faint mRNA signal was detected in hair bulb material, Dr Commo, like Tobin and colleagues, demonstrated TRP-2 expression in hair follicle-derived melanocytes *in vitro*. By contrast, melanogenic melanocytes located in the epidermis of these donors expressed TRP-2. Whether TRP-2 expression is present in hair follicle melanocytes producing predominantly pheomelanin fibers is not clear. One possible implication of this finding is that the TRP-2 associated rearrangement of L-dopachrome to DHICA to produce DHICA melanins is less critical here than in the epidermis, or that in hair bulb melanocytes may exhibit the spontaneous rearrangement of L-dopachrome leads to 5,6-dihydroxyindole alone. Moreover, the absence of TRP-2 in this compartment may make hair bulb melanocytes less resistant to the cytotoxic action of decarboxylated indoles. The basis for the species difference (i.e. mouse vs human) in TRP-2 expression in hair bulb melanocytes is not immediately clear, although it is interesting to note that the levels of TRP-2 in extracts of human melanocytic cells have previously been described as being relatively low compared with those in murine melanocytes (Kameyama *et al*, 1995).

Hormonal Regulators of Hair Follicle Melanogenesis

The search for local regulators of follicular melanogenesis has generated significant interest among pigment cell biologists, particularly those that fluctuate during the hair cycle and are coupled to the early anagen onset of melanogenesis in bulbar melanocytes. Pro-opiomelanocortin (POMC) products have been proposed as the main regulators of follicular pigmentation (Tobin and Paus, 2001; Kauser *et al*, 2004; Slominski *et al*, 2004b). Studies in guinea-pigs have shown that α -melanocyte stimulating hormone (MSH) increased the proportion of black to gray hairs when administered intramuscularly. But the injection of both α -MSH and a potent synthetic analog [Nle4,D-Phe7]- α -MSH into human skin results in increased melanogenesis particularly of sun-exposed skin. No effect, however, was seen in hair follicles. We have recently found that the expression of α -MSH is very low to undetectable in pigmented hair bulb melanocytes versus epidermal counterparts both *in vivo* and *in vitro* (Kauser *et al*, 2005).¹ But it is likely that the melanocortin 1-receptor (MC1-R), the product of the extension

(e) locus and cognate receptor of α -MSH, is an important positive regulator of hair pigmentation. This G protein-coupled membrane receptor is activated upon binding of POMC-derived ACTH and α -MSH peptides (cf. Slominski *et al*, 2004a, b). The resultant signal transduction cascade results in the activation of adenylate cyclase activity, subsequent cAMP production, and results in increased melanocyte proliferation, melanogenesis, and dendrite formation.

The expression and translation of POMC gene products are expressed in the skin in a hair cycle-dependent manner, being low at telogen and high during anagen development (cf. Slominski *et al*, 2004a, b). In murine skin, the expression of ACTH, α -MSH, β -endorphin, and MC1-R fluctuates during cycling, suggesting that these peptides may be involved in the reconstitution of the follicle pigmentary unit and perhaps also in the regulation of hair growth itself. There is particular interest in the pro-eumelanogenic peptide α -MSH because mutations in extension (e) [MC1-R] or agouti (a) [ASP] loci affect murine follicular melanocytes. Moreover, accumulation of POMC products in scalp outer root sheath follicular keratinocytes in the scalp and less so in the overlying epidermis suggests that the activity of the local POMC/MC1-R axis plays an important role in the physiologic regulation of anagen-associated hair pigmentation. It is noteworthy that MC1-R gene polymorphisms have been strongly linked to red hair and fair skin in humans (cf. Tobin and Paus, 2001; Slominski *et al*, 2004b).

The existence of a POMC complementary system, namely the β -endorphin/ μ -opiate receptor system (see Tobin and Kausser in this issue, pp. x–x), has recently been shown to influence human epidermal and follicular melanocyte biology by inducing changes in melanocyte dendricity, proliferation, and melanogenesis (Kausser *et al*, 2004). Dr Söbia Kausser (Dr Desmond Tobin's Group) at this workshop presented a study examining the role of corticotrophin-releasing hormone (CRH)—the most proximal element of the hypothalamic–adrenal axis (HPA) and chief regulator of pituitary POMC gene and peptide expression—in human hair follicle biology and in hair pigmentation. She showed that CRH can modulate follicular melanocyte behavior and possibly also hair growth. There is increasing evidence to suggest that mammalian skin contains an equivalent of the HPA axis, composed of locally produced CRH that together with signaling via CRH receptors (CRHRs) 1 and 2 may regulate skin homeostasis (Ito *et al*, 2005; Slominski *et al*, 2004a). Dr Kausser presented evidence that specific CRH and urocortin analogs, designed to variably activate CRH-R1 or -R2, can have variable effects on melanogenesis, dendricity, and proliferation.¹

Other positive regulators of melanogenesis include the bone morphogenic proteins (BMP). Originally discovered as an osteo-inductive extract derived from bone matrix, the BMP consist of a family of dimeric proteins (BMP-2–15) that are secreted as signaling molecules to regulate several aspects of cutaneous development and function via their participation in cell proliferation, differentiation, and death. BMP and their associated downstream transcription factors also influence neural crest-derived cell populations, including melanocytes, in several species. BMP-2 specifically targets tyrosinase gene expression in primary quail neural crest cultures resulting in increased melanin synthesis.

Furthermore, BMP-4 may stimulate proliferation of normal human melanocytes in culture, likely via the proliferation-associated receptor, BMPR-IA. BMP-4 treatment of human cutaneous melanocytes reduces the expression of mRNA and protein for tyrosinase and the tyrosinase activator PKC β . Production of BMP-4 is greater in melanocytes than keratinocytes, suggesting that this BMP may be an autocrine factor for melanocytes. At this workshop Dr Michael Fessing (Dr Vladimir Botchkarev's Group) reported hair pigmentation abnormalities in Noggin transgenic mice (Sharov *et al*, 2003). Overexpression of Noggin, an antagonist of BMP-2 and BMP-4, in agouti mice resulted in a wide range of phenotypes including accelerated hair follicle development, upregulation of keratinocyte proliferation and downregulation of apoptosis in the hair bulb. In addition, these mice exhibited increased eumelanogenesis in hair bulb melanocytes that replaced the normal agouti pheomelanogenesis that occurs in wild-type mice. Thus, the switch between eumelanogenesis and pheomelanogenesis may, in part, be regulated by the BMP signaling pathway. There is also some evidence that BMP-4 can stimulate proliferation and inhibit melanogenesis in cultured human epidermal melanocytes.²

Melanocytes in the epidermis express BCL-2, an antiapoptotic oncogene associated with cell survival. Follicular melanocytes, especially those located in the outer root sheath, also express this survival factor. It has been proposed that BCL-2 inhibits cell death particularly in areas where reactive oxygen species are generated (e.g., melanogenesis) by regulating anti-oxidant pathways. Moreover, UV-induced apoptosis of melanocytes *in vitro* can be blocked via nerve growth factor-induced upregulation of BCL-2 (cf. Slominski *et al*, 2004a). Nitric oxide (NO) is a potential source of oxidative stress in skin. At this workshop Ms Heather Sowden (Dr Desmond Tobin's Group) presented a comparative assessment of NO synthase (NOS) expression in the pigmentary units of the human scalp epidermis and hair follicle during the hair cycle. NO, a lipophilic free radical, is synthesized from the oxidation of L-arginine by NOS. NOS include the constitutive isoforms eNOS and nNOS which produce nM amounts of NO to exert physiologic roles, e.g. vasodilation/neurotransmission, and the inducible iNOS that produces NO in mM amounts in response to stimuli like LPS and is associated with both beneficial and detrimental effects. Moreover, a common tissue marker of radical-mediated damage is nitro-tyrosine (reaction of NO with superoxide anion (peroxynitrite)) expression. Recently, a role for NO in cutaneous physiology has been advanced including in vasodilation, wound healing, and melanogenesis (cf. Slominski *et al*, 2004a). Ms. Sowden showed that epidermal melanocytes *in situ* express both eNOS and bNOS (but not iNOS) whereas no eNOS or nNOS could be detected in hair bulb melanocytes during anagen (Sowden *et al*, 2005).³ The constitutive NOS isoforms were expressed, however, in weakly pigmented and/or amelanotic melanocytes of the outer root sheath

¹Kausser *et al* (submitted).

²Yar M, Park HY, Botchkarev V, Stewart K, Panova I, Gilchrist BA: Bone morphogenetic protein 4 modulates melanocyte proliferation and melanogenesis. *J Invest Dermatol* 119:1;337, 2002 (abstr).

and sebaceous gland. Similar compartmentalization was also evident for nitrotyrosine expression. These opposing expression patterns in the epidermal and follicular pigmentary units may reflect UVR stimulation of melanocytes in the epidermis but not those in the proximal hair bulb. Interestingly, NO synthase and nitrotyrosine expression was modulated by the hair growth cycle. There are several potentially important implications of these findings. For example, there is evidence that eNOS-derived NO may protect against apoptosis, for example via the induction of bcl-2 expression. Thus, eNOS expression is highest in apoptosis-resistant epidermal melanocytes but low/undetectable in hair bulb melanocytes that can be susceptible to apoptosis. Thus, different compartments of the skin and hair follicle may have different regulation of the cutaneous NOS/NO system (Sowden *et al*, 2005).²

In conclusion, several difference aspects of hair follicle melanocyte behavior were featured in this very interesting workshop at the 2004 IMHRS meeting, which add to our steady dissection of differences between these two cutaneous pigment cell populations. It is likely that exploitation of these differences will enable us to differentially regulate activity of these melanocyte sub-types to influence pigmentation of the skin and hair follicle. The chairs would like to thank all the speakers for their stimulating presentations and the audience for their active participation.

DOI: 10.1111/j.1087-0024.2005.10117.x

Manuscript received September 20, 2004; revised November 22, 2004; accepted for publication December 2, 2004

Address correspondence to: Prof. Desmond J. Tobin, Department of Biomedical Sciences, University of Bradford, Bradford, West Yorkshire BD7 1DP, UK. Email: dtobin@bradford.ac.uk

Copyright ©2005 by The Society for Investigative Dermatology, Inc.

References

- Botchkareva NV, Botchkarev VA, Gilchrist BA: Fate of melanocytes during development of the hair follicle pigmentary unit. *J Invest Dermatol Symp Proc* 8:76–79, 2003
- Botchkareva NV, Khlgatian M, Longley BJ, Botchkarev VA, Gilchrist BA: SCF/ckit signaling is required for cyclic regeneration of the hair pigmentation unit. *FASEB J* 15:645–658, 2001
- Commo S, Bernard BA: Melanocyte subpopulation turnover during the human hair cycle: An immunohistochemical study. *Pigment Cell Res* 13:253–259, 2000
- Hibberts NA, Messenger AG, Randall VA: Dermal papilla cells derived from beard hair follicles secrete more stem cell factor (SCF) in culture than scalp cells or dermal fibroblasts. *Biochem Biophys Res Commun* 222:401–405, 1996
- Ito N, Ito T, Kromminga A, *et al*: Human hair follicles display a functional equivalent of the hypothalamic–pituitary–adrenal (HPA) axis and synthesize cortisol. *FASEB J* 2005 June 9 [Epub ahead of print]
- Kameyama K, Sakai C, Kuge S, *et al*: The expression of tyrosinase, tyrosinase-related proteins 1 and 2 (TRP1 and TRP2), the silver protein, and a melanogenic inhibitor in human melanoma cells of differing melanogenic activities. *Pigment Cell Res* 8:97–104, 1995
- Kausar S, Thody AJ, Schallreuter KU, Gummer CL, Tobin DJ: Beta-endorphin as a regulator of human hair follicle melanocyte biology. *J Invest Dermatol* 123:184–195, 2004
- Kausar S, Thody AJ, Schallreuter KU, Gummer CL, Tobin DJ: A fully functional proopiomelanocortin/melanocortin-1 receptor system regulates the differentiation of human scalp hair follicle melanocytes. *Endocrinology* 146:532–543, 2005
- Nishikawa S, Kusakabe M, Yoshinaga K, *et al*: *In utero* manipulation of coat color formation by a monoclonal anti-c-kit antibody: Two distinct waves of c-kit-dependency during melanocyte development. *EMBO J* 10:2111–2118, 1991
- Nishimura EK, Jordan SA, Oshima H, *et al*: Dominant role of the niche in melanocyte stem-cell fate determination. *Nature* 416:854–860, 2002
- Peters EM, Maurer M, Botchkarev VA, Jensen K, Welker P, Scott GA, Paus R: Kit is expressed by epithelial cells *in vivo*. *J Invest Dermatol* 121:976–984, 2003
- Peters EM, Tobin DJ, Botchkareva N, Maurer M, Paus R: Migration of melanoblasts into the developing murine hair follicle is accompanied by transient c-Kit expression. *J Histochem Cytochem* 50:751–766, 2002
- Sharov AA, Weiner L, Sharova TY, *et al*: Noggin overexpression inhibits eyelid opening by altering epidermal apoptosis and differentiation. *EMBO J* 22:2992–3003, 2003
- Slominski A, Tobin DJ, Shibahara S, Wortsman J: Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiol Rev* 84:1155–1228, 2004a
- Slominski A, Wortsman, Plonka PM, Schallreuter KU, Paus R, Tobin DJ: Hair follicle pigmentation. *J Invest Dermatol* 124:13–21, 2004b
- Sowden HM, Naseem KM, Tobin DJ: Differential expression of nitric oxide synthases in human scalp epidermis and hair follicle pigmentary units: Implications for the regulation of melanogenesis. *Br J Dermatol* 153:2005 (in press)
- Tobin DJ, Paus R: Graying: Gerontobiology of the hair follicle pigmentary unit. *Exp Gerontol* 36:29–54, 2001
- Tobin DJ, Slominski A, Botchkarev V, Paus R: The fate of hair follicle melanocytes during the hair growth cycle. *J Invest Dermatol Symp Proc* 4:323–332, 1999
- Tobin DJ, Kausar S: Beta-endorphin: The forgotten hair follicle melanotropin. *J Invest Dermatol Symp Proc* 10:212–216, 2005
- Yoshida H, Hayashi S, Shultz LD, Yamamura K, Nishikawa S, Nishikawa S, Kunisada T: Neural and skin cell-specific expression pattern conferred by steel factor regulatory sequence in transgenic mice. *Dev Dyn* 207: 222–232, 1996



The Technology

The Revâge670 Laser System

In the mid 1960s, Professor Endre Mester, a medical doctor and researcher, began investigations into the subject of laser radiation and cancer. Along with some of his colleagues at the Institute for Pathological Anatomy and Experimental Cancer Research, Mester questioned the concept of the existence of a link between laser irradiation, the biological effects and cancer development in heretofore healthy mice. His research uncovered no such link, but rather demonstrated that red laser light accelerated the re-growth of hair on the shaved skin of the mice.

This was a small but germinal piece of work. Many other researchers have followed Mester's work and we now find ourselves in the very dynamic field of LLLT and hair growth in humans.

This technology is unlike almost all other laser technology that is used in the medical industry. By definition, Low-Level Laser Therapy (LLLT) does not raise the body temperature above 36 degrees centigrade. The biological effects of this type of irradiation are purported to be the result of increases in cellular metabolism. In the case of hair growth, it is believed that the proliferating cells of the dermal papilla are "rehabilitated", thereby reverting to healthy, anagen follicle producing optimal quality hair shafts.

The Revâge670 Laser System incorporates all the ergonomic features required to optimize therapy and employs a proprietary algorithm that maximizes efficacy vis-à-vis laser radiation. The Revâge670 Laser System contains thirty 4-milliwatt lasers affixed in a rotating helmet. The result is a simple and effective therapy. The only variable that must be considered when delivering therapy is skin phototype. The more darkly pigmented subjects receive more therapy than the less pigmented subjects. The fluence delivered per unit of time remains unchanged.

Research Opportunities with Apira

At Apira, we are leading the way with well-designed, solid clinical research programs. We have made a commitment to work with the very best physicians and scientists available. To that goal, we encourage all like-minded professionals to contact us for participation in the research programs.

Please address all correspondence and telephone calls to:

Mr. Morgan Pepitone
Vice President of Operations, Apira
100 Bayview Circle, Suite 2200
Newport Beach, CA 92660
Tel. 949.854.9900
Fax 949.854.9922



APIRA – Science In Rejuvenation

100 Bayview Circle, Suite 2200 • Newport Beach, CA 92660

Phone: 866.982.7472 • Email: info@apriascience.com

