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From In-Vitro to In-Vivo: Corporate Development and Efficacy of a Topical Hair Growth Agent Derived from Natural Extracts

Kelly Michael Glynn
Grand Valley State University

FROM IN-VITRO TO IN-VIVO: CORPORATE DEVELOPMENT AND EFFICACY OF A TOPICAL HAIR GROWTH AGENT DERIVED FROM NATURAL EXTRACTS

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

By

Kelly Michael Glynn

To

Biology Department
Grand Valley State University
Allendale, Michigan
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Stultum est in luctu capillum sibi evellere, quasi calvito maeror levaretur

"It is foolish to pluck out one's hair for sorrow, as if grief could be assuaged by baldness"

Marqus Tullius Cicero
Tusculanarum Disputationum (III, 26)
(http://www.worldofquotes.com/topic/Hair/index.html)

This work is dedicated to Vicki, for her love, encouragement, unwavering support and patience; to my entire family, especially my mom, without whom I never could have journeyed this far; and to Sam, Abbie and Hunter who were my motivation to finish.

Love always!

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ABSTRACT

FROM IN-VITRO TO IN-VIVO: CORPORATE DEVELOPMENT AND EFFICACY OF A TOPICAL HAIR GROWTH AGENT DERIVED FROM NATURAL EXTRACTS

by Kelly Michael Glynn

Androgenetic alopecia (male pattern baldness) affects up to 50% of the world's population, propelling the development for a possible treatment. The hair follicle is influenced by several genetic and physiologic factors, which, when gone awry, lead to androgenetic alopecia. Vascular endothelial and keratinocyte growth factors are believed to be promoters of hair growth, as is inhibition of the proteasome complex. The cytokine IL-1α is also known to regulate follicle dynamics. The research objective described herein was an attempt to develop a botanical blend, which could mediate the above biomarkers, be successfully incorporated into a safe topical product and be evaluated for *in-vivo* efficacy. By using an arbitrary scoring system to evaluate in-vitro performance, botanical extracts were screened in cell culture and enzyme assays. A Design of Experiments analysis, utilizing analyses of variance and multiple linear regressions, was performed to derive an optimized blend of Lichochalcone, Saw Palmetto, Shiso and Green Rooibos for incorporation into the prototype formulation. After passing human irritancy and sensitization testing, these extracts were coupled with liposomes to create a final prototype that was also screened for long-term stability. The end product was used in a clinical-type trial, assessing its effectiveness to increase scalp

hair density, promote anagen follicle activity and increase the growth rate of the hair fiber. The product was benchmarked by Rogaine® Extra Strength (5% minoxidil) and Rovisomes Biotin (commercially available). The twelve-week study involved sixty-nine males experiencing varying degrees of androgenetic alopecia who underwent ¼" length haircuts and a series of digital imaging focusing on a transition zone area of interest. The three test products significantly increased hair density and the number of anagen follicles compared to baseline values. Growth rate was up regulated for users of the prototype and Rogaine®. Subjective self-assessment of the products revealed the prototype to be the least effective in improving hair quality characteristics, but with no significant difference to the other two products. These results indicate the herbal blend of Lichochalcone, Saw Palmetto, Shiso and Green Rooibos, in a liposomal base, has the potential to be an effective topical treatment for androgenetic alopecia.

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A.) INTRODUCTION

From a non-biological view point, hair appears to be nothing more than a "symbolic and psychosocial" (Hadshiew, et al., 2004) body part which can be modified to bolster self-image and self-esteem from both individual and societal perceptions and interactions (Stenn and Paus, 2001; Paus and Foitzik, 2004). In fact, it has been stated, "the psychological importance of hair to man is in inverse ratio to its physical function" (Ebling, 1976). Unbeknownst to many, human hairs are the only bodily appendage which can be manipulated to influence societal relationships and improve self-image (Stenn and Paus, 2001). These two attributes have lead to the creation of a multi-billion dollar industry (Paus and Cotsarelis, 1999) which attempts to aid men and women with their coiffed appearance.

Embedded within the skin, however, is a biological marvel that imparts the physical characteristics of length, color, shape and diameter to the hair fiber, which is so important to the mirror and society. This marvel is the hair follicle, which retains embryonic cues to help create hair fibers throughout an individual's life (Legue and Nicolas, 2005). It does so in a cyclical process in an asynchronous fashion at the level of the individual follicle (Stenn and Paus, 2001). Therefore, each human hair follicle can be viewed as an independent biological entity that determines whether or not a visible hair fiber is present. Through a series of intricate mechanisms, many of which are still unknown, hair follicles can become quiescent beyond normal cyclical patterns leading to diseased states generically termed alopecias. Aside from purely genetic manifestations of

baldness or aggressive infection, states of baldness such as alopecia areata (Cotsarelis and Millar, 2001; Mulinari-Brenner and Bergfeld, 2001), telogen effluvium, chemical/psychological induced baldness (Cotsarelis and Millar, 2001), and androgenetic alopecia are all considered temporary states (Mulinari-Brenner and Bergfeld, 2001).

Contrary to perception, however, hair serves several critical biological functions. These include defense against insects, camouflage, thermal regulation, sensory detection, skin cleansing, and signal transporters (Stenn and Paus, 2001). Additional roles for hair include ultraviolet protection and screens to prevent intrusion of foreign particles into critical membranes such as the eye. Sexual communication is also influenced by hair, or the lack thereof, in events such as sexual selection in mating preference, identification of puberty in adolescents and markers of masculinity in the appearance of chest, pubic and beard hair (Camacho, et al., 2000).

Since hair on all different parts of the body can serve multiple biological functions, the creation and development of the hair fiber must then have its own biological apparatus operating under unique controls. Again, this apparatus is the hair follicle (Camacho, et al., 2000), and despite its uniqueness, its multi-mechanistic and wondrous operation is concealed from the naked eye. Visibly, the only confirmation of its existence is the emergence of the keratinized hair fiber protruding from the epidermal surface. When this evidence is no longer present, or when the rate of its regeneration capacity begins to diminish, the impact on humans can be monumental, despite being painless and non-life threatening. Secondary side effects of hair loss include psychological and emotional stress, shame, embarrassment, depression, loss of confidence and self-worth, perception of age, and lack of societal acceptance (Hadshiew,

et al., 2004). These effects have been known to be especially significant in younger aged men who suffer from some form of hair loss (Girman, et al., 1998), but both genders can experience hair loss in some form (Camacho, et al., 2000). So, hair serves a physiological, psychological and cosmetic role.

The premise for conducting hair research is to understand the biological mechanisms that drive fiber growth and fulfill the accessory roles of this fibrous appendage, and how such mechanisms may relate to other anatomical and physiological processes. From a purely cosmetic standpoint, however, understanding how hair grows, and uncovering the hope of how it may be restored in the case of baldness in humans, has led to a race to be the first to claim success, even if marginal, at reversing a complex set of interactions which ultimately create this void on the human scalp. A prominent researcher in hair growth, Dominique Van Neste, has extensively researched the biological phenomenon and cultural impacts of hair. He states:

> As grooming may be controlled by genetic factors it seems no surprise that hair has probably been a material of interest since the very early days of mankind. Engravings on the wall of caves and pre-historical sculptures provide the earliest representations of hair and clearly tell us about its symbolic dimensions. Hair- on the scalp and on the body – is communication. It conveys messages about ourselves, it tells how we interconnect with social codes and status. Hair is everywhere! (Van Neste, 2003).

B.) EMBRYOLOGY OF HAIR

Hair follicle development begins in-utero, at two to three months, on the eyebrows, lips, chin, and nose, with later development occurring on the back, abdomen, and limbs. Follicle formation appears to be mediated in waves, with distance to the preceding follicle being a determinant for new follicle placement. As a result, each follicle maintains its own cycle (Serri and Cerimele, 1990). A precursor to follicle

formation, however, is the establishment of a connective network between epidermal and mesenchymal tissues. Signal transmission from mesodermic tissue to embryonic ectoderm causes thickening of the ectoderm and formation of the hair placode, which emits return signals to the underlying mesenchyme, causing it to condense. Following mesenchymal condensation, epithelial placode cells proliferate downward into the mesenchymal tissue eventually forming the dermal papilla (Kulessa, et al., 2000). The bulbous dermal papilla is the control center from which all-future hair growth regulation and cycling will originate (Paus and Foitzik, 2004).

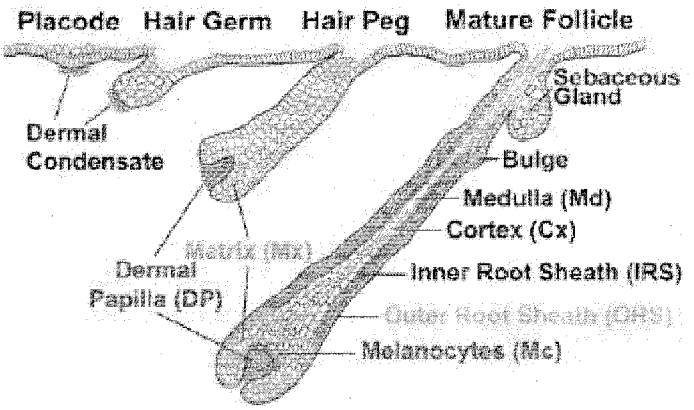


Figure 1- Development of the Hair Follicle From In-Utero to Maturity (used with permission from Dr. Elaine Fuchs 01/08/2009; Rendl et al., 2005, Fig. 1A, p. 1911)

With the basic hair follicle in place, surrounding keratinocytes will begin differentiating (Kulessa, et al., 2000) to form eight different concentric layers of the follicle. These include: the outer root sheath (ORS) and its companion layer; Henle's and Huxley's layers; cuticle; and the medulla, cortex and cuticle of the actual hair fiber (Paus and Foitzik, 2004).

The intimate signaling during follicle embryogenesis between the epithelial and mesenchymal layers drives subsequent hair growth stages in post-natal life. Botchkarev and Kishimoto (2003) state:

> Extensive interactions between these two embryologically different hair follicle compartments lead to the formation of the hair shaft producing mini-organ that shows a cyclic activity during postnatal life with periods of active growth and hair shaft formation (anagen), apoptosis-driven involution (catagen), relative resting and hair shedding (telogen-exogen).

The continual cross talk between the ectoderm derived epidermis and mesoderm derived mesenchyme (Serri and Cerimele, 1990) ultimately produces a distinct hair fiber. This cross-talk is achieved through a combination of: genetic factors (Birch and Messenger, 2001; Midorikawa, et al., 2004; Ishimatsu-Tsuji, et al., 2005); a plethora of molecular signaling (Hoffman, et al., 1996; Kulessa, et al., 2000; Botchkarev, et al., 2001; Botchkarev and Kishimoto, 2003); stem cell activity (Alonso and Fuchs, 2003; Blanpain, et al., 2004; Legue and Nicolas, 2005; Kim, et al., 2006; Zhang, et al., 2006); neuroimmunoendocrine circuitry (Paus, et al., 2006); innervation (Hordinsky and Ericson, 1996); hormones (Thornton, et al., 1993; Hamada, et al., 1996; Ellis, et al., 1998; Choi, et al., 2001); and vascularization (Lachgar, et al., 1996; Lachgar, et al., 1998; Sordello, et al., 1998; Yano, et al., 2001). The sum of these mechanisms results in approximately five million hair follicles body-wide, with an estimated 80,000-150,000 follicles dispersed throughout the human scalp (Krause and Foitzik, 2006). After birth, this number does not increase, whereas the size and shape of each follicle can (Paus and Cotsarelis, 1999).

Initial fetal hair is termed lanugo hair and is typically shed at eight months in-utero, replaced with additional lanugo hairs that last into the fourth month post-partum. Vellus unpigmented, fine, short hairs, replace the secondary lanugo hairs, and typically cover a majority of the skin surface. Through a prolonged continuation of the above mechanisms certain regions of vellus hairs are transformed into terminal hairs, which are

thicker, longer and pigmented (Jankovic and Jankovic, 2004). The transformation of terminal hairs back to vellus hairs on the scalp, and the underlying physiological changes occurring within the follicle, is the trademark of androgenetic alopecia.

C.) HAIR ANATOMY AND PHYSIOLOGY

The hair follicle can be divided into three regions: biological synthesis, keratinization and the hair fiber portion. Biological synthesis occurs at the hair bulb, which encompasses the dermal papilla (Robbins, 1994) and the matrix (Krause and Foitzik, 2006). Mesenchyme-derived dermal papilla cells cue the surrounding epithelial-derived matrix cells to undergo mitosis during active growth (Philpott, et al., 1990), proliferating at one of the highest rates in the human body, even outpacing some forms of cancers (Camacho, et al., 2000; Krause and Foitzik, 2006). Deposition of melanin from melanocytes embedded within the matrix (Paus and Cotsarelis, 1999) also occurs resulting in coloration of the cortical cells of the emerging hair fiber. As epithelial cells continue to proliferate, they are pushed upward toward the skin surface, where they enter the matrix-derived inner root sheath (IRS). The IRS consists of three distinct layers: Henle's layer, Huxley's layer, and IRS cuticle. Henle's layer contains keratinized sheath cells that help form a scaffold to support interior structures. Huxley's layer, interior to Henle's layer, along with the IRS cuticle, forms a rigid tube (Camacho, et al., 2000) that confers shape to the upward migrating cells and ultimately shapes the hair fiber (Paus and Cotsarelis, 1999). The cells continue to elongate as they enter the zone of keratinization. Sulfur transport, mainly in the form of cysteine, into the cells results in the formation of disulfide bonds, and eventual keratin synthesis, imparting strength to the hair fiber. Keratin synthesis continues until the cell is nearly filled with the fibrous material. As a

result, transcription and translation cease, nuclear degradation occurs, and the cell is dehydrated. As this process occurs throughout the hair shaft cells, the fiber takes on its final shape and diameter (Camacho, et al., 2000; Robbins, 1994). As the hair fiber finally emerges from the scalp, it is classified as a "keratin appendage from a follicle which is embedded in the dermis," composed of dehydrated cuticle, cortical and medullary cells held together by biological cements (Robbins, 1994).

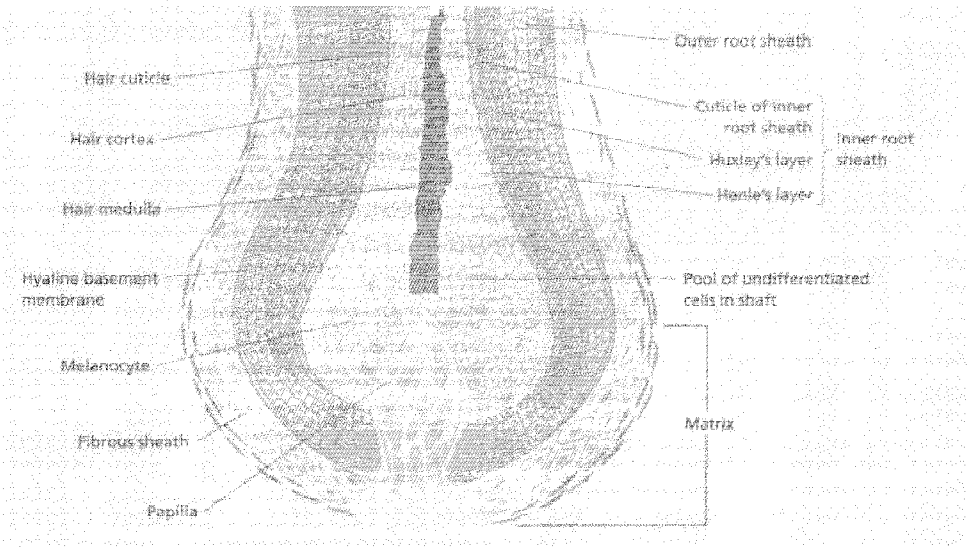


Figure 2- Anatomical Structure of the Mature Hair Follicle (http://www.pg.com/science/haircare/hair_twh_13.htm)

The aforementioned cuticle is the outermost covering of the hair fiber, formed from an interlocking interaction with IRS cuticles (Serri and Cerimele, 1990). Cuticle cells are similar in length to cortex cells, but lack the cortex cells' elasticity, and impart chemical and mechanical resistance as well as moisture regulation to the hair fiber. The cortex cells, derived from proliferating matrix, contain interwoven, keratin filaments, which resemble a coiled structure microscopically. This longitudinal arrangement offers great elastic properties to the hair fiber, compared to protective cuticle cells (Camacho, et al., 2000). The medulla is the innermost section of the hair fiber, and is derived from

apical dermal papilla cells. In scalp hairs, the medulla is often labeled as not present, but in reality, these cells are often porous (Robbins, 1994) and highly vacuolated, making their appearance unnoticeable (Camacho, et al., 2000). An additional structure, which is involved in constructing the hair fiber and regulating follicle cycling events, is the outer root sheath (ORS). The ORS surrounds the hair follicle in its entirety, from the uppermost portion in the epidermis remaining with the permanent hair follicle, all the way down to the dermally imbedded hair bulb (Serri and Cerimele, 1990). The ORS is epithelial-derived, and throughout its length appears to have a multitude of functions advantageous for skin repair as well as hair physiological activities. From potential harboring of sebaceous glands and epidermal stems cells, to secretion of multiple cellular-forming constituents, the ORS offers the skin repair mechanisms following injury and/or damage. In addition, the ORS structure contains melanocytes (for coloration), Langerhans' cells (for immune response) and Merkel cells (for neurological response), all of which are involved in skin restructuring or aiding the hair follicle to respond to infection or sensory stimuli (Paus and Cotsarelis, 1999). External to the ORS, and enclosing and separating the entire follicle from the skin epithelial layer, is a membrane consisting of extracellular proteins (Rendl, et al., 2005).

Biological components associated with and/or surrounding the hair follicle include: the apocrine gland (perspiration); the sebaceous gland (lipid synthesis and secretion); the isthmus (site of sensory fibers); the arrector pili muscle (sympathetic nerve fibers synapsed with smooth muscle cells to aid in thermal barrier responses); the infundibulum (the region, along with the hair canal, spanning the skin surface to just above the sebaceous gland, representing the first body structures to be keratinized); and

the bulge (a stem cell repository) (Kanitakis, 2002; Serri and Cerimele, 1990).

D.) STEM CELLS AND THE HAIR FOLLICLE

As concisely summarized by Kolf et al, the stem cell niche:

> encompasses all of the elements immediately surrounding the stem cells when they are in their native state, including the non-stem cells that might be in direct contact with them as well as ECM (extracellular matrix) and soluble molecules found in that locale. All of these act together to maintain the stem cells in their undifferentiated state. It is assumed that certain cues must find their way into the niche to signal the stem cells that their differentiation potential is needed for the regeneration or repopulation of a tissue (Kolf, et al., 2007).

For the hair follicle, the bulge is the stem cell niche, supporting both hair regeneration and the skin epithelium (Alonso and Fuchs, 2003) and marks the end of the permanent hair follicle (Serri and Cerimele, 1990). The portion of the hair follicle inferior to the bulge undergoes remodeling processes throughout the hair cycles. Downward growth into the dermis, during active growth, is accomplished through epithelial-mesenchymal communications between the permanent and regenerating portions of the follicle. Derived from and residing in the ORS, the bulge was found to retain its relative position of origin in individuals ranging in age from two weeks to twenty-one years (De Viragh and Meuli, 1995).

Since the dermal papilla is referred to as the command center of the hair follicle and is surrounded by highly proliferating matrix cells, initial hypotheses stated hair follicle stem cells should logically reside somewhere in the same vicinity (De Viragh and Meuli, 1995). In work done as early as 1994, however, evidence pointed to stem cell-like activity occurring from a region in approximation to the arrector pili muscle (Rochat, et al., 1994). Further research verified the bulge was the location harboring stem cells to be used to regenerate the active growth stage for the hair follicle, and possibly for aiding in

epidermis repair following tissue damage, since these epithelial stem cells can reproduce sebaceous glands and skin layers when these components are destroyed (Paus and Foitzik, 2004). Furthermore, when the colony-forming ability of keratinocytes isolated throughout the hair follicle was examined, cells isolated from the bulge were able to generate 95% of the colony-forming cells in culture, while only 5% of colony-forming cells were attributed to the matrix region. Also, the colony forming keratinocytes showed no increase in growth potential when co-cultured with papilla fibroblasts, an indication that there may be a certain level of independence between the two follicle cell types and how they regulate hair fiber growth (Kobayashi, et al., 1993).

Within the basal layer of the epidermis, stem cells also reside, which through division, upward movement, and terminal differentiation result in mature skin cells. It has been suggested that the bulge stem cells may be the multi-potent progenitors of the epidermal stem cells since: epidermal stem cells have no identifiable niche within the epidermis; bulge stem cells exhibit slower cycling times than epidermal stem cells; and retention of radioactive thymidine is longer within bulge cells, indicative of the cells not undergoing rapid cell cycle/mitosis events. Daughter cells derived from bulge stem cells eventually differentiate to assist in the formation/maintenance of the hair follicle matrix, the sebaceous gland, and the basal layer of the epidermis (Alonso and Fuchs, 2003).

A prominent theory as to how the bulge drives hair follicle regeneration is known as the bulge activation hypothesis. The premise is the mesenchymal dermal papilla emits a signal to the bulge stem cells, which in turn, begin sending stem cells to the hair bulb region. These mobile signal carriers create rapidly dividing keratinocytes, which will form/reform the hair bulb, and through a series of additional stages, new hair fibers are

produced (Alonso and Fuchs, 2003; Camacho, et al., 2000; Stenn and Pause, 2001). The length of that fiber will correspond to the number of cell divisions taking place, and when the proliferative capability of these is reached, catagen induction begins (Camacho, et al., 2000).

Recent research has also indicated the matrix stem cells, derived from the bulge, are highly organized and compartmentalized with each sector responsible for forming a certain portion of the hair fiber. At the inner core of the matrix, multipotent stem cells reside which produce daughter cells that give rise to transient progenitors of the various structures of the hair fiber. These progenitors are in the layer external to the multipotent stem cells. The third and final concentric layer consists of post-mitotic ancestors of the transient cells, whose function is to construct the columns of the hair bulb which will be used to construct the different components of the hair fiber. This organizational pattern creates a radial distribution within the bulb. How cells align along the central vertical axis of the bulb determines each cell's fate, giving rise to the IRS, hair fiber cuticle or the medulla (Legue and Nicolas, 2005).

Stem cells are necessary for maintaining cellular and physiological balance and also for initiating repair mechanisms and tissue regeneration following wounding. For hair growth regulation, stem cells and their niche are critical for re-initiating the active growth phase in the hair cycle. For this reason, characteristics of the bulge and its stem cells include: 1.) The bulge is anatomically formed postnatally following the initial growth stage; 2.) Basal layers within the bulge are attached to a basement membrane, while several genes, responsible for producing cytoskeletal, extracellular matrix, cell adhesion molecules and proteins are active within; 3.) The niche housing the stem cells

within the bulge is partitioned asymmetrically; 4.) Isolated stem cells are able to reproduce several generations of clones in culture, as well as reproduce hair follicles and sebaceous glands; 5.) Stem cells respond to external cues to initiate regeneration events; 6.) Once in the hair bulb region, stem cells undergo specific spatial organization to properly reconstruct the hair follicle and eventual hair fiber. As a whole, these attributes indicate the bulge is a pertinent player in hair growth and skin function (Blanpain, et al., 2004; Legue and Nicolas, 2005).

CHAPTER II: HAIR CYCLES

A.) ANAGEN, CATAGEN, TELOGEN & EXOGEN

One of the most interesting aspects of the hair follicle is its cyclic nature, divided among stages of active fiber growth (anagen), growth cessation and apoptosis of the temporary follicle (catagen), and a period of rest and/or remodeling (telogen) (Muller-Rover, et al., 2001; Paus and Foitzik, 2004; Rendl, et al., 2005; Robbins, 1994). As technology has advanced to observe follicle morphogenesis, and the complexity of these cyclic events has come to be understood, the hair follicle has become "an attractive system for studying major biological phenomena" (Stenn and Paus, 2001).

Anagen begins with the cues to start the reconstruction of the follicle bulb and ends when active growth ceases and additional cues are received to begin the deconstruction of the same bulb. Anagen occurs in six distinct steps (Müller-Röver, et al., 2001), hallmarked by the rapid proliferation of matrix cells, active melanin deposition via melanocytes and keratinization of epidermally progressing cells, all contributing to the emergence of the characteristically distinct hair fiber (Robbins, 1994; Stenn and Paus, 2001). The actual molecular cues, which initiate this growing process, remain obscure; however, the anatomical events occurring to lead to active growth resemble those events happening during embryonic follicle development. Epithelial cells divide in a downward fashion to reach the dermis, where dermal papilla cells anchor to a basement membrane. Upon reaching their end point, growth begins in an upward and outward fashion with the development of the IRS and hair shaft (Stenn and Paus, 2001).

The processes initiating anagen include trauma and/or wounding of the hair follicle, hair plucking, and chemical influence. Merely cutting the hair fiber does not

induce anagen events (Stenn and Paus, 2001). In addition, the same cellular and molecular pathways involved during embryonic follicle development are believed to take part in the governing of anagen initiation and progression throughout life (Cotsarelis and Millar, 2001).

Approximately 80-90% of a human's scalp hairs are in the anagen stage (Robbins, 1994), which lasts between two to five years. The factor that determines the length of a single hair fiber then is the duration of time the follicle spends in the anagen phase (Mulinari-Brenner and Bergfeld, 2001), while the diameter of a hair fiber is determined by the size of the dermal papilla. A larger dermal papilla generally contains more proliferating matrix cells. The dermal papilla volume is created during the first stages of anagen (Cotsarelis and Millar, 2001). The shape of the hair fiber is ultimately determined by the shapes of the ORS and IRS, through which the upward migrating cells are funneled (Camacho, et al., 2000). In many cases, the anagen portion of the hair cycle will function normally in the scalp through approximately ten progressions, which corresponds to roughly forty years of age (Krause and Foitzik, 2006).

All of the work done during anagen is destroyed during the eight stages of catagen (Müller-Röver, et al., 2001) defined as "highly controlled involution of the hair follicle resulting in apoptosis and terminal differentiation" (Krause and Foitzik, 2006). The characteristic signs of apoptosis (cell shrinkage, blebbing, nuclear condensation, and eventual cell fragmentation) are all observed in cells of the follicle (Botchkareva, et al., 2006). The switch from anagen to catagen is again somewhat of a mystery in terms of causation, molecular signaling and genetics, but certain events, such as chemical application, trauma, and environmental factors have been found to invoke this regressive

phase (Stenn and Paus, 2001).

During catagen, the temporary portion is disassembled by means of regulated cell death occurring in certain follicle structures. In addition, the dermal papilla separates from the follicle bulb (Stenn and Paus, 2001). The size of the entire follicle is also diminished, as is the position of the follicle. Follicles may reach into the subcutaneous fat layer during anagen, while the same post-catagen follicle transcends into the dermal tissue (Cotsarelis, 1997).

One of the most interesting aspects of apoptosis during catagen is it occurs in waves, beginning with the area of melanin deposition in the bulb, spreading to the hair matrix, then the ORS and IRS, and finally converging on the hair shaft (Botchkareva, et al., 2006). Even more intriguing, however, is dermal papilla fibroblasts do not undergo apoptosis, nor do most bulge cells, at anytime during the hair cycles. This demonstrates that these cell types are critical for future regeneration events in subsequent growth cycles (Botchkareva, et al., 2006; Cotsarelis, 1997).

Considering the highly controlled state of catagen, a relatively small percentage of scalp hairs are in this stage at any given time, roughly 1-2% (Robbins, 1994). Furthermore, it is expedient, lasting anywhere from three to six weeks (Mulinari-Brenner and Bergfeld, 2001). The catagen events of the hair follicle are "to delete the old hair shaft factory and to bring the inductive machinery of the cell to a point where a new follicle can form, utilizing once again, the stem cells of the bulge and the inductive powers of the papilla" (Stenn and Paus, 2001).

The cycle in which most hairs spend their time, second to anagen, is telogen. This is the resting phase of the follicle, or as alternately proposed, a "pre-regeneration" state of

anagen (Camacho, et al., 2000), or an anagen brake (Stenn and Paus, 2001). By the time a follicle has entered into telogen, epithelial keratinocytes have surrounded compacted dermal papilla fibroblasts, which have minimal proliferative activity (Paus and Foitzik, 2004). Even though follicle activity has diminished in telogen, relative to anagen and catagen, the follicle still contains the necessary cell populations to generate a new follicle and fiber in the next anagen cycle. These include epithelial stem cells, ORS keratinocytes, and melanocytes (Camacho, et al., 2000). The dermal papilla is terminal to and adjacent to the hair germ (the base of the quiet follicle) and the entire structure is located in approximation to the arrector pili muscle, well into the dermal layer (Camacho, et al., 2000; Stenn and Paus, 2001). Residing in the hair shaft is the club hair, or dead hair (Paus and Cotsarelis, 1999) to which the root sheaths have attached. The bulb, matrix and dermal papilla are now separated from the shaft, effectively preventing any further growth of the hair fiber. The club hair will eventually be shed, in the cycle known as exogen, which is believed to be distinct from the anagen cycle, even though the two events can occur simultaneously. A club hair will still reside within the follicle, while a new anagen phase has started rebuilding the lower, temporary portion of the regenerative follicle. In fact, it has been proposed, the cycle of exogen encompasses the factors that anchor the club hair into the follicle, and what molecular events take place to release the club hair from the shaft (Stenn and Paus, 2001). On average, an individual loses anywhere from 50-100 hairs per day as a result of the exogen event (Robbins, 1994).

The percentage of scalp follicles in telogen ranges from 10-20% (Robbins, 1994), and lasts, on average, three to nine months (Camacho, et al., 2000). From a historical research perspective, little investment of time has been put into telogen research, and as

such, the magnitude of molecular biomarkers involved in this stage is still unknown (Stenn and Paus, 2001).

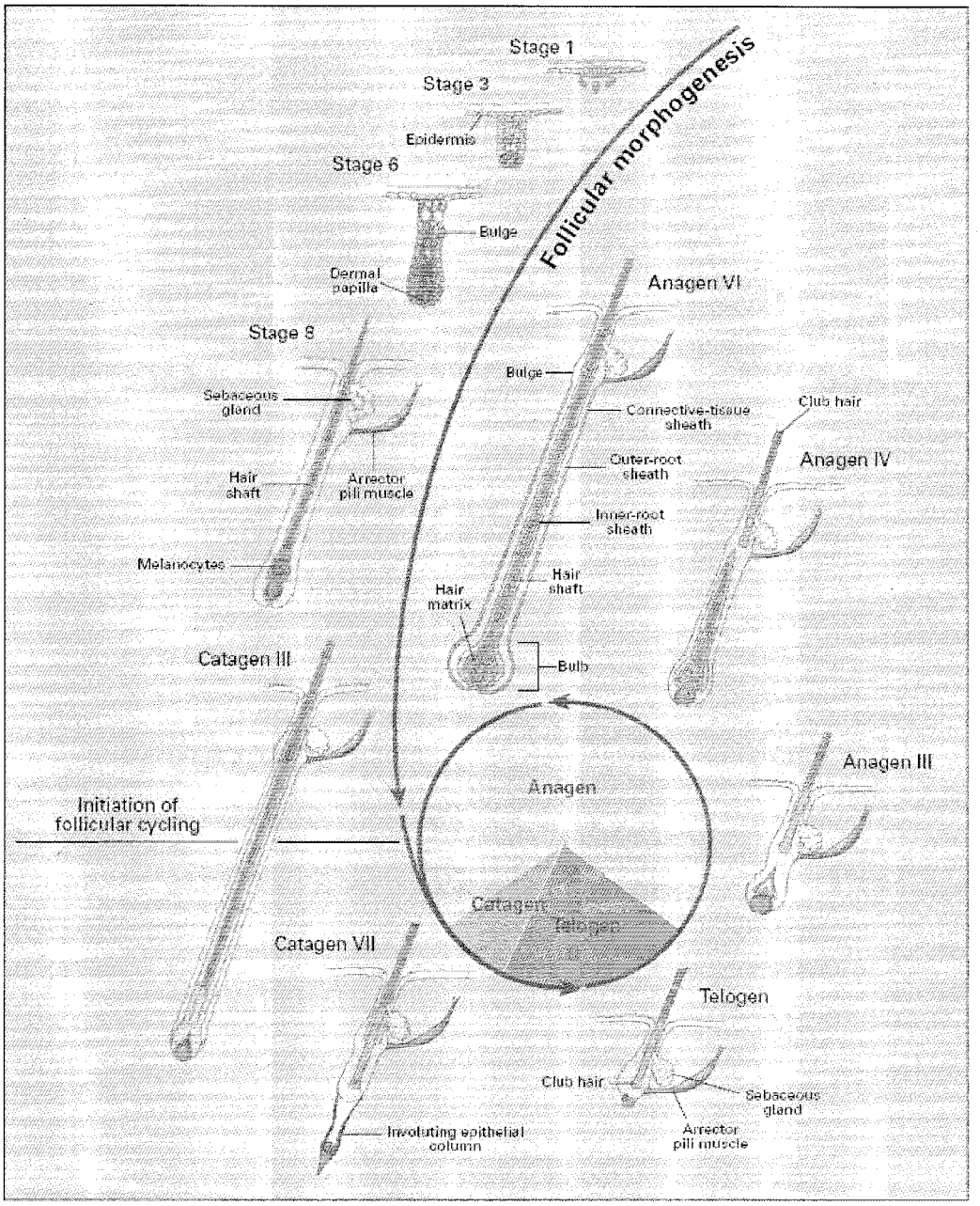


Figure 3- Progression and cyclic nature of hair follicles (used with permission from the New England Journal of Medicine 01/12/2009; Paus and Cotsarelis, 1999, Fig. 2, p. 493.)

B.) TYPES OF HAIR ABNORMALITIES

It is apparent the alterations in the basic operations of the hair cycles will lead to some type of hair disorder. In addition, since the number of follicles an individual possesses is an embryogenic determined entity, the mechanisms of operation in each follicle will determine hair abnormalities (Mulinari-Brenner and Bergfeld, 2001). For example, if anagen is excessively prolonged, hypertrichosis or hirsutism can result, both of which are excessive hair growth disorders. When the anagen cycle is continually shortened during successive cycles, or if telogen is extended, alopecia will result. Different associated factors can drive a multitude of other hair disorders, including alopecia areata (patchy hair loss either on the scalp or throughout the body caused by an autoimmune malfunction); anagen effluvium (sudden shedding of actively growing hair as is observed in chemotherapy patients); telogen effluvium (abnormal number of follicles induced into telogen often due to certain drugs/medications or fever); permanent alopecia (entire follicle is destroyed due to infection, autoimmune disorders or skin cancers); and androgenetic alopecia (influenced by the androgenic steroids testosterone and dihydrotestosterone [DHT] resulting in characteristic balding patterns on the scalp). With the exception of genetic deletion and follicle organ deletion, malfunctions in one or more of the cycling events, or aberrant factors and/or influences involved within a cycle, will result in an abnormal manifestation of hair growth or hair loss (Hamada and Randall, 2006; Hibberts, et al., 1998; Mulinari-Brenner and Bergfeld, 2001, Paus and Cotsarelis, 1999).

The remainder of this review will focus mainly on androgenetic alopecia (AA), which is the most prevalent form of hair loss on the scalp (Hoffman, 2003) accounting for

approximately 95% of those individuals suffering from some type of hair growth defect (Choi, et al., 2001). It is estimated between 40-50% of the world's population is afflicted with this disease (Krause and Foitzik, 2006; Robbins, 1994) with up to eighty million Americans experiencing hair loss (Leavitt, 2003). It can affect both males and females (Mulinari-Brenner and Bergfeld, 2001) and children as young as six years of age have also been known to be susceptible (Tosti, et al., 2005). In regards to intervention, AA is a non-permanent form of baldness (Paus and Cotsarelis, 1999) since the follicle does remain embedded within the dermis and continues to cycle even in the absence of a visible hair fiber (Mulinari-Brenner and Bergfeld, 2001). The biological complexity of the hair follicle in terms of operational control is still a mystery waiting to be solved, while the physiological function in humans has taken on a largely cosmetic role. The hope for restoring hair growth remains limited with surgical repair via hair transplantation being the only permanent fix to date (Leavitt, 2003). Hope for better success, by easier means, will come if only the mystery of biological complexity is unraveled. Understanding these mechanisms and the influences associated with them may eventually lead to that cosmetic milestone.

CHAPTER III- FACTORS INVOLVED IN HAIR GROWTH/LOSS

A.) HORMONES

Two major contributing factors to AA are androgens and genetic predisposition (Ellis, et al., 1998). When the synthesis of androgenic steroids begins with cholesterol, several weaker intermediate hormones are produced which can and are converted to more potent forms via enzymes. The major androgenic steroid circulating throughout the body is testosterone, while the still more potent steroid is dihydrotestosterone (DHT) converted from testosterone by the enzyme 5α-reductase (5α-R). Androgen potency is determined by its binding affinity to the androgen receptor (AR) within a cell's cytoplasm. A further dimension to consider is free-circulating androgens. Sex-hormone-binding-globulin (SHBG), binds almost 70% of available testosterone, while albumin takes hold of another 19%. That leaves approximately 10% of available testosterone as free circulating hormone. It still remains unclear as to whether or not bound testosterone, onto either protein, can be active. Once an androgen binds the AR, the complex shuttles to the nucleus where it exerts its effects via gene transcription or suppression. The primary androgens related to hair follicle physiology are testosterone and DHT, though additional hormones and factors can certainly play a role in the regulation of hair growth/loss. These include levels of available hormones, levels of conversion enzymes, the number of androgen receptors present within a cell and/or tissue and the influence of the androgen complex on genes directly involved in hair growth modulation (Hoffmann, 2003).

Some of the most interesting research conducted on hair growth regulation has been the realization that androgens, particularly DHT, have different modulatory properties throughout the body and even on the scalp (Hibberts, et al., 1998; Hoffmann,

2003; Stenn and Paus, 2001; Thornton, et al., 1993). For instance, the occipital scalp is androgen insensitive, while the frontal, parietal and coronal scalps are all androgen sensitive. The scalp vertex is androgen sensitive but androgen independent. The axillary portions of the body are androgen dependent whereas the eyebrows and eyelashes are androgen insensitive (Stenn and Paus, 2001). So even though a majority of the body is covered by hair, either terminal or vellus, not all hairs are affected in the same way by androgens.

Extensive research has been done in an attempt to explain how different body locales respond to androgens. Work done by Hibberts, Howell and Randall (1998) found the number of androgen receptors in dermal papilla fibroblasts was significantly higher in follicles extracted from balding scalp tissue compared to non-balding scalp tissue, while the androgen binding affinity from both regions was identical, as was the protein content of both receptors. Such a discovery implies the number of androgen receptors in a given area may have a significant impact on hair growth. However, expression of androgen receptor mRNA has also been found throughout the hair follicle in both balding and non-balding individuals (Asada, et al., 2001), indicating more is needed than just a large quantity of androgen receptors to precipitate a balding condition.

Additional factors influencing the expression and/or control of the AR include phosphorylation of specific serine residues in the AR protein. When androgen is bound to the AR, phosphorylation of these residues increases. Attention to the phosphorylation of serine 213 in the AR protein is especially intriguing since it may be involved in certain developmental processes, and has been shown to promote the degradation of the AR (Taneja, et al., 2005). The discovery of isoformic co-activators for AR transcription has

also hinted at explaining the different responses to androgens throughout body tissue. The short isoform, ARA70β, was observed only in the dermal papilla portion of the follicle, and its expression was reduced in balding tissue compared to non-balding tissue (Lee, et al., 2005). It is interesting to surmise that the co-activator for the AR is upstream from AR transcription. Simultaneous expression of both the co-activator and receptor protein could limit hair growth in the different scalp tissues by negatively regulating dermal papilla proliferation signals.

When beard dermal papilla cells (androgen dependent and sensitive) were compared to non-balding scalp dermal papilla cells (androgen independent and/or insensitive) for the conversion of testosterone to DHT, via uptake of radiolabled testosterone, it was discovered the beard cells only converted testosterone to DHT, not the scalp cells. So not only is the AR important for hair growth modulation, but the presence of the converting enzyme, 5α-R, is also a critical factor (Thornton, et al., 1993).

The 5α-R enzyme has two isoforms, 5α-R1 and 5α-R2, which have been found to have specificity within the cell as well as in tissue activity (Stenn and Paus, 2001). Epithelial cells of the hair follicle have an abundance of 5α-R1, compared to a limited amount of 5α-R2, while the dermal papilla contains mRNA for 5α-R2 almost exclusively. The quantities for both of these isoforms were not different in balding and non-balding cases (Asada, et al., 2001). Since the dermal papilla appears to solely express 5α-R2 mRNA, and is the command center of the actively growing hair follicle, it seems reasonable to believe the 5α-R2 isoform is an essential enzyme for androgen metabolization where androgen influence plays a significant role in hair growth (Asada, et al., 2001; Hoffmann, 2003), and that the main site for androgen activity is in the

dermal papilla of the hair follicle (Hamada, et al., 1996).

Additional research examined the levels of androgens in hair from the different zones (balding versus non-balding) of the scalp. Serum levels of androgen were also tested. Levels were compared within individuals and to controls (non-balding subjects). Vertex DHT was higher in balding subjects versus non-balding individuals, but no real difference in DHT levels was found to exist between balding and non-balding zones from the same subject. Serum levels of both DHT and testosterone were also higher in balding participants compared to non-balding participants (Bang, et al., 2004).

Besides testosterone and DHT, estrogen and estrogen intermediates are also involved in hair regulation in both males and females. Ohnemus et al., (2006), in their review of estrogen function, state: "estrogens and estrogen metabolism are at least as important as androgens in male and female hair biology." Reasons listed for this premise include inhibition of hair re-growth in mice when estrogen is applied topically, which directly opposes the common practice of topical application of estrogens for hair growth stimulatory effects in women suffering from androgenetic alopecia. Such an anomaly points to another complex mode of influence, further compounded by species specificity. In addition, research is also referenced which points to estrogens having the capability of squelching androgen metabolism, even in the dermal papilla, to the point that the amount of DHT produced, following testosterone stimulation, is reduced. The enzyme aromatase, which can convert testosterone to the less potent 17β-estradiol, has also been isolated from cells which have active AR expression occurring, suggestive of a complex regulatory role between the two hormone classes, which when gone awry, may be manifested in some hair disorder. This complexity is further complicated by research

showing hormone receptors of the different classes can communicate with each other, leading to alterations of the individual hormonal cascades and regulation of gene expression (Ohnemus, et al., 2006).

The hormone prolactin and its receptor were also found to exist in human hair follicles, and that treatment with exogenous prolactin inhibited cultured follicle growth, while endogenous prolactin, and its receptor, expression increased as follicles advanced into the catagen cycle. The region of prolactin activity appears to be limited to epithelial cells in the follicle since mesenchyme derived dermal papilla cells exhibited no presence of the hormone, or its receptor (Foitzik, et al., 2006).

In terms of hormones, the hair follicle exhibits a high degree of complexity, just compounding the difficulty of understanding how this miniaturized organ operates. Even though common male patterned baldness bears the moniker of the androgen steroid, much more is in play in the regulation of hair growth, from conversion enzymes to intermediates to expression locations.

B.) GENETICS AND GENES

Due to the high degree of integration between the different physiological systems regulating hair growth, and how the phenotype or clinical appearance can manifest itself over time, balding and non-balding conditions are polygenetic traits, which are reached upon on a threshold crossing of genetic events gone awry. Birch and Messenger (2001) examined first and second generation inheritance patterns for balding and non-balding males. After five hundred seventy-two men were studied, the researchers concluded: balding is common in Caucasian males; with increasing age, baldness also increases regardless of what stage the balding pattern is; if a balding condition manifests itself

before the age of thirty, the probability is high for the father of these individuals to also be bald; if men live long enough, they will go bald; males who are resistant to balding typically come from non-balding families; and the female balding condition possesses a higher threshold state since androgens contribute to baldness and are typically at lower levels in females.

Several gene expression profile studies have been conducted comparing the balding and non-balding traits. Macroarray research that examined expression levels of 1185 genes, ranging in function from cell cycle regulation to apoptosis, discovered nearly ten percent [107] of the genes were alternately expressed in balding subjects. Genes involved in signal transduction and cell cycle regulation both had decreased expression levels in dermal papilla cells from balding sites. Furthermore, fourteen growth factor genes also exhibited decreased expression. Taken together, these findings indicate the balding follicles were functionally inactive (Midorikawa, et al., 2004).

A microarray analysis comparing male and female gene expressions revealed 1436 genes were common to both genders, while ninety-seven genes showed differential expression between the two sexes, with a majority [89] at higher levels in males, and only eight were positively up regulated in females. What state of balding the test subjects were in was not mentioned, but since hair fiber extractions included the upper portion of the ORS sheath, follicles were most likely in the anagen cycle (Kim, et al. 2006).

A murine microarray study was done, whose hair follicle cycles can be synchronized by epidermal depilation via waxing. As the hair cycle stages progressed, expression patterns were compared to non-depilated skin tissue. Some key findings of this research show twenty-three days post depilation, expression patterns returned to

baseline levels relative to the non-depilated tissue expressions. Following the hair removal event, genes involved in inflammation response and anagen initiation were the main factors, while in the mid-late anagen cycle, keratin related genes were active, all of which correspond to the regenerative events of hair fiber formation (Ishimatsu-Tsuji, et al., 2005).

In regards to specific gene events, Ellis, Stebbing and Harrap (2001), analyzed androgen receptor gene polymorphisms in balding and non-balding males. They determined a restriction fragment length polymorphism, *StuI,* residing in exon one of the AR gene, "is a necessary, but not sufficient component of the polygenic predisposition to male pattern baldness." In young men with baldness, 98% percent of the subjects possessed this marker, while older, balding males also exhibited polymorphism to a high degree. Those without this particular polymorphism were not likely to go bald. In cases where the marker was present, but baldness was not, the balding threshold may not have been reached yet, and/or the other required polygenetic dispositions were not present in those individuals. This was later corroborated on a different ethnic group, which showed the dual band polymorphism in balding males resulted from a single nucleotide base change (adenine to guanine) in the first exon of the X chromosome (Levy-Nissenbaum, et al., 2005).

The 5α-Reductase gene would also be a logical target for researchers hoping to understand the genetics involved in baldness and/or hair growth regulation. Since the 5α-reductase enzyme has two isoforms, there are two separate genes as well. SRD5A1 codes for the 5α-R1 enzyme, and is located on chromosome five, whereas SRD5A2 codes for the 5α-R2 form and is on chromosome two. What research has uncovered though, is both

genes have elevated expression levels in the frontal scalp for both men and women, but no significant difference in distribution patterns could be discerned for both genes between balding and non-balding states. The implication here is the 5α-R gene or enzyme variability is not a contributing factor to a balding disposition. In addition, since sons of balding fathers also tended to display baldness in this study, the likelihood of a simple X-linked mode of inheritance appears unlikely. With all factors considered, a polygenetic mechanism produces the balding phenotype (Ellis, et al., 1998; Levy-Nissenbaum, et al., 2005).

The *hairless* gene, when defective in mice, will produce normal looking hair follicles at birth. When the first catagen cycle occurs, however, the entire mouse becomes bald with dermal cysts forming in the follicle. In regards to humans, alterations in *hairless* leads to an entire body devoid of hair (Camacho, et al., 2000). The dermal papilla is separated from the hair shaft and improper catagen deconstruction of the follicle results in loss of recovery for future hair growth events. Another human hair deficiency, identified as MIM: 601705, is synonymous with expression of the recessive *nude* phenotype in mice. Despite the presence of a hair follicle, the hair shafts cannot break the epidermal barrier, resulting in complete baldness (O'Shaughnessy and Christiano, 2004).

When the gene expression profiles are tallied, there is supporting evidence for over one hundred seventy-nine genes, involved in hair growth functions (Ishimatsu-Tsuji, et al., 2005; Kim, et al., 2006; Midorikawa, et al., 2004) and at least one hundred different proteins expressed in the hair follicle (O'Shaughnessy and Christiano, 2004). Such a large number just re-emphasizes the complexity of the network controlling this

"miniaturized" organ and the uncertainty of which factor(s) is absolutely essential for initiating the process of hair loss. Through a thorough examination of genetic analysis, molecular factors, environmental influences, and inferential implications from both in-vitro and animal studies, the intricacy of the hair follicle may be solved. Even then it is still unknown if it can be manipulated in order to restore the proper balance ensuring a cosmetically, socially and mirror image pleasing perception and appearance.

C.) MOLECULAR MARKERS

Since the hair follicle has come to be understood as a complex mini-organ, it has garnered much interest "for studying major biological phenomena" (Stenn and Paus, 2001) in addition to hair biology research. Cell cultures of hair follicle keratinocytes and dermal papilla fibroblasts are now commercially available, and isolation and culturing techniques of surgically extracted follicle cell types are well established (Havlickova, et al., 2004; Stenn and Paus, 2001; Randall, 1996; Warren and Wong, 1994; Philpott, et al., 1990; Buhl, et al., 1989; Lattanand and Johnson, 1975). The use of such cell types and cultures, along with animal models, have aided in identifying at least eighty-five growth factors, transcription factors, cytokines and various protein and receptor constituents involved in hair growth regulation (Stenn and Paus, 2001).

Two of the growth factors involved with the anagen stage of hair follicle (HF) cycling are vascular endothelial growth factor (VEGF) and keratinocyte growth factor (KGF). VEGF expression from hair follicle dermal papilla cells (HFDPC) is believed to promote vascularization to the re-developing hair bulb during the telogen-anagen switch (Lachgar and Moukadiri, et al., 1996). This vascularization is believed to be a reconnection of blood vessel networks (angiogenesis) (Yano, et al., 2001), which has

been found to be active during anagen stages (Mecklenburg, et al., 2000). VEGF is a critical growth factor for initiating angiogenic events (Lachgar, et al., 1999; Lachgar, et al., 1998; Shweiki, et al., 1993) and minoxidil treatment on human dermal papilla cells showed up-regulation and elevated expression of VEGF mRNA and protein (Lachgar, et al., 1998).

KGF, also known as fibroblast growth factor-7, is produced by HFDPCs and has been shown to mediate keratinocyte proliferation, with maximal expression occurring during the anagen V sub-stage in murine studies, which corresponds to a period of rapid hair fiber synthesis (Kawano, et al., 2005). Elevated expression levels of KGF receptor mRNA has been observed in rat embryo follicugenesis, while injections of recombinant KGF resulted in marked hair growth, as did keratinocyte proliferation and follicle hypertrophy (Danilenko, et al., 1995). KGF is also an attractive research growth factor target since it potentially involves mesenchymal-epithelial cross talk-HFDPC secretions acting on keratinocytes and affects hair morphology in KGF null mice (Guo, et al., 1996; Finch, et al., 1989), which is prevalent in HF dynamics.

Inhibition of the proteasome has also been found to be a stimulatory mechanism for hair growth, with targeting of the same molecular markers initiating bone-remodeling events. Specifically, compounds that can suppress proteasome function suggestively promote hair follicles to enter the anagen hair cycle phase, thereby promoting hair fiber growth. The proposed mechanism by which this occurs is through mediation of the Hedgehog, Bone Morphogenic Protein (BMP) and Wnt signaling cascades. Even topical application of aldehydic proteasome inhibitors induced anagen and resulted in significant hair growth versus a negative control (Mundy, et al., 2007). Sonic hedgehog is active

during embryonic development of the follicle (Dlugosz, 1999) and has been found to be critical for subsequent hair fiber synthesize (Paladini, et al., 2005; St. Jacques, et al., 1998). The presence of Wnt signaling leads to activated β-catenin and target gene transcription (Huelsken and Behrens, 2002), which has been shown to promote the switch from telogen follicles to anagen follicles and prolonged anagen function (Huntzicker and Oro, 2008). Furthermore, it has been shown β-catenin activates VEGF, associated in angiogenesis, in certain colon cancers (Levy, et al., 2002), an element already revealed as pertinent to hair growth.

Expression of the cytokine IL-1α is limited to HF keratinocytes, including those in the HF matrix (Hoffmann, et al., 1997; Xiong and Harmon, 1997; Philpott, et al., 1996). The exact role of IL-1α in HF dynamics is still elusive since some studies have demonstrated inhibitory events (Hamada, et al., 2003; Mahe, et al., 1996) while more recent research portrays a positive influence for up-regulating known hair growth initiating factors (Boivin, et al., 2006).

D.) CLINICAL METHODS

The use of liposomes in hair loss treatment applications has grown, since it has been shown that topical delivery of liposome-based formulations can penetrate into the skin with selective targeting to the HF (Jung, et al., 2006). Incorporated into the vesicles can be a wide range of active ingredients, varying in size and hydrophilicity (Li and Hoffmann, 1997; Li and Hoffmann, 1995) that could potentially influence specific molecular biomarkers and/or cellular constituents within HF structures. These markers could include VEGF, KGF, IL-1α and the proteasome. Incorporating botanical extracts into liposomes, to treat androgenetic alopecia, is an attractive methodology to compete

with the only two approved Food and Drug Administrations treatments, Propecia and Rogaine® (Sawaya, 1998). In addition, by using botanicals, the negative aspects of using animal-derived ingredients, availability issues, range of effectiveness (Aburjai and Natsheh, 2003) and reduced costs, relative to total health care costs (Saikia, et al., 2006) are minimized. Furthermore, the drive to develop hair loss treatments will be propelled by diminished male self-image resulting from hair loss, increasing hair loss in females and an increasing aging population (Euromonitor, 2007).

From a current clinical perspective, the methods available to study hair loss/growth patterns involve modifications of the classic trichogram ("forceful hair pluck", Sperling, 1991) enhanced by epiluminescence photography and digital imaging, aptly named the phototrichogram. The primary parameters typically followed in hair loss interventions are hair density and hair fiber diameter. Secondary, are the hair growth rate and the anagen-telogen ratio (Hoffmann, 2001). Successful topical hair growth treatments should be able to either individually or in combination: 1.) Arrest miniaturization of the follicles; 2.) Promote terminal hair formation while reducing vellus hairs; 3.) Promote actively growing hairs (anagen); 4.) Enhance the growth rate of actively produced hair fibers and/or increase hair fiber diameters (Hoffmann and Van Neste, 2005).

E.) RESEARCH OBJECTIVE

As discussed, the complexity of hair growth regulation and malfunction is intricate. Testing all aspects would be time consuming and cost prohibitive from a corporate perspective, whose main goal is to develop, market and sell an efficacious

product. As a result, the hair research team originated a multi-faceted bioassay screening approach, targeting established and novel factors associated with hair growth and loss.

The research objective described in detail in the following pages was three-fold. First, several botanical extracts were screened for in-vitro efficacy of affecting specific biomarkers and the proteasome, pertinent to hair growth regulation. These markers are the growth factors VEGF and KGF and the cytokine IL-1α. To our knowledge, this four-fold examination of cellular constituents, in response to a vast array of herbal extracts, is a unique approach to researching hair loss/growth potential remedies. The optimal performing extracts were then analyzed through a Design of Experiments (DOE) to understand effective concentration ranges and synergistic effects. The second objective involved incorporating the select blend of herbal components into a liposomal-based hair growth promoting topical solution that meets human safety criteria and product stability performance. Upon successful completion, the final objective was to conduct a semi-clinical trial testing this developed herbal-liposomal based product on actual androgenic alopecia subjects following modified phototrichogram protocols, using minoxidil (Extra Strength Rogaine®) as the benchmark, with comparison to a proprietary liposomal blend already clinically tested.

CHAPTER IV- MATERIALS AND METHODS

All cell culture preparation and cell treatment was carried out using standard aseptic techniques in laminar flow-through hoods. All extracts were tested in duplicate on the same plate, and at least one additional trial was conducted using a different culturing and/or passage of cells, botanical extract concentration and/or reagents/kit lots. The screening of extracts and DOE analyses occurred from February 2005-June 2006.

A.) VEGF AND KGF ASSAYS

Mesenchymal-derived human Hair Follicle Dermal Papilla Cells (HFDPC) and HFDPC Growth Medium were purchased from Cell Applications (San Diego, CA, USA). HFDPCs were stored in liquid nitrogen until flask seeding, at which time, 15mL of HFDPC medium was dispensed into a 75 -cm^2 (growing surface area) collagen coated flask (BD BioSciences, Bedford, MA, USA). A single, frozen cryovial (>500,000 cells) was thawed in a 37°C water bath for one minute, and the contents dispensed into the prepared flask, followed by a 1-mL medium rinse of the cryovial. The newly seeded flask was placed in a 37°C / 5% CO_2 incubator overnight. Following cell attachment, the HFDPC medium was replaced with fresh medium and cells were allowed to reach 80-90% confluency (approximately one week). HFDPCs were then plated onto either 24-well or 96-well, bovine collagen type 1 (BD BioSciences, Bedford, MA, USA) coated plates after a Hanks Balanced Salt Solution without Ca^{2+}/Mg^{2+} rinse (Fisher Scientific, Hanover Park, IL, USA) and detachment with trypsin/EDTA (Fisher Scientific, Hanover Park, IL, USA). Cells were plated at concentrations of 35,000 cells/well, 500-µL of medium (24-well plates) or 7,500 - 10,000 cells/well and 200-µL of medium (96-well plates). Cell attachment was allowed to occur overnight in a 37°C / 5% CO_2 incubator.

B.) IL-1α ASSAY

Proliferating human epithelial-derived keratinocytes (HEK001) were purchased from ATCC (Manassas, VA, USA) and stored in liquid nitrogen until seeding. Filter-sterilized culture medium was prepared with Invitrogen's Keratinocyte Serum Free Medium-1x (Carlsbad, CA, USA), supplemented with 1% L-glutamine (Invitrogen), 1% penicillin and 1% amphotericin b (Mediatech, Manassas, VA, USA) and 0.1-0.2 μg/mL Gibco Bovine Pituitary Extract (Carlsbad, CA, USA) and 17 ng/mL of Invitrogen's epidermal growth factor. A frozen cell vial (600,000 cells) was thawed in a 37°C water bath for one minute and its contents were transferred to a centrifuge tube containing 9.0-mL of HEK001 medium. Following centrifugation at 1200 rpm for 10 minutes, the resulting pellet was re-suspended in new culture medium and transferred to a Corning 75 cm^2 flask (Corning, NY, USA), where 80-90% confluency was reached in a 37°C / 5% CO_2 incubator (3-4 days). Upon reaching the desired confluence, HEK001s were rinsed with Hanks Balanced Salt Solution with Ca^{2+}/Mg^{2+} and then detached with trypsin/EDTA. A HEK001 rinse medium (Keratinocyte serum free medium, 1% amphotericin b, 1% penicillin and 10% Fetal bovine serum [Hyclone, Logan, UT, USA]) was used to transfer cells to a centrifuge tube where pellet formation occurred at 1200 rpm for 10 minutes. Supernatant aspiration was followed by addition of 30mL culture medium and plating HEK001 occurred in 24 well cell plates (200,000 – 600,000 cells / 500 μL) or 96 well cell plates (32,000 cells / 200-220 μL). Cell attachment was allowed to occur overnight in a 37°C / 5% CO_2 incubator.

C.) EXTRACT EFFICACY TESTING

Botanical extracts were tested on plated HFDPC and HEK001 cells for VEGF, KGF and IL-1α (DOE only). Briefly, extracts were prepared to a 50-mg/mL concentrated stock solution using dimethyl sulfoxide (Fisher Scientific, Fairlawn, NJ, USA), 99.5% ACS ethyl alcohol (Acros, NJ, USA) and purified water at 50%, 30% and 20% levels. Solvent concentrations were adjusted as appropriately for each extract's solubility properties. Extract solutions were vortexed thoroughly and sonicated in a 23°C water bath for 10 minutes. Test solutions were prepared for each extract by diluting with the proper medium type under aseptic conditions. Each extract was tested in-vitro at end concentrations of 10 µg/mL, 1.0 µg/mL and 0.1 µg/mL. Cell culture plates were removed from incubation and examined under an inverted microscope (Cambridge Instruments, Buffalo, NY, USA) to ensure appropriate cell morphology and attachment to the culture plate. Under aseptic conditions, media was carefully aspirated and replaced with the proper amount of prepared extract. For controls, cells were treated with the same volume of respective media only. After addition of the extract, cell culture plates were returned to 37°C / 5% CO_2 incubation for 24 hours. Following incubation, cell supernatant was removed from the cell culture wells and transferred to vials for freezing at -20°C until ELISA analysis.

D.) ELISA TESTING

HFDPC and HEK001 cell supernatants were evaluated for their concentration of VEGF/KGF and IL1α, respectively, by ELISA. Protocols for KGF (R&D Systems, Minneapolis, MN, USA), VEGF (BioSource, Camarillo, CA, USA) and IL-1α (BioSource) were followed according to the manufacturers' instructions, with the

exception of centrifugation of supernatants to eliminate particulates (due to minimal volumes and clear nature of the supernatant). Colorimetric readings from ELISA plates were measured on either the Wallac Victor^2 1420 Multilabel Counter (Turku, Finland) or the Perkin-Elmer multilabel plate reader (Waltham, MA, USA). Amount of expressed growth factor or cytokine was expressed as pg/mL and percent expression relative to the control within each plate.

E.) PROTEASOME ASSAY

Boston BioChem's (Cambridge, MA, USA) SDS Activation Format assay was utilized to evaluate the ability of botanical extracts to suppress the 20S subunit activity, the catalytic core of all proteasome isoforms, in cell-free systems. Activity is measured by fluorescence readings of solutions, to which a peptidyl substrate is added, following core activation by sodium dodecyl sulfate. Botanical extracts were prepared the same as they were in the cell culture assays, but diluted to test concentrations with reaction buffer provided in the assay kit. One hundred μL of diluted extracts were added to 96-well Nunc (Rochester, NY, USA) black-wall and side multi-dish plates, followed by active enzyme solution (0.0998% end concentration). Diluted substrate was then added (0.1% end concentration) to the wells and the plates were incubated at ambient, but dark, conditions for thirty minutes. Fluorescence was measured with the Perkin-Elmer plate reader, set to an excitation wavelength of 360 nm and emission wavelength of 465 nm. All samples were run in triplicate, including the positive controls (enzyme, substrate and reaction buffer), negative controls (substrate and reaction buffer only) and reaction buffer only. Influence of proteasome activity was determined by percentage of fluorescence relative to the positive control.

F.) EVALUATION OF BOTANICAL EXTRACT PERFORMANCE

An arbitrary scoring system was developed to summarize the performance of each extract for each assay. The scores were summed and extracts exhibiting elevation of VEGF and KGF (126% - >300% of the control) and suppression of proteasome activity (89% - <30%) were considered possible candidate botanical ingredients for a hair growth-promoting product. Positive scores for each assay were deemed desirable in terms of biomarker performance, and the score ranges were based upon the history of maximal extract performance (up regulation of VEGF and KGF, relative to media controls; suppression of the proteasome complex relative to positive control performance of the proteasome and substrate) observed in early bioassay work. These selected extracts were then analyzed through Fusion Pro Design of Experiments (DOE) software (S-Matrix Corporation, Eureka, CA, USA) layouts based on testing of fixed ranges of each extract, either in combination or as a single ingredient. Each DOE analysis incorporated multiple, individually prepared replicates as a means to determine standard error and statistical analysis included multiple linear regression and ANOVA. Two DOEs were performed, with each measuring growth factor expression (VEGF and KGF), cytokine expression (IL-1α) and proteasome activity through the appropriate cell treatment/assay methods described above. Each DOE analysis compared performance as percent relative to the control in each assay, as well actual expression levels (pg/mL) for each biomarker. DOE 1 consisted of five botanical extracts whereas DOE 2 evaluated the performance of only four extracts. A follow-up ingredient optimization study was also conducted, via DOE set-up and analysis, to arrive at a finalized ingredient testing concentration.

G.) INGREDIENT FINALIZATION

The selected extracts were incorporated into a series of prototype bases to mimic potential topical delivery. A maximal extract containing prototype formulation was submitted for human Repeat Insult Patch Testing through TKL Research Incorporated (Rochelle Park, NJ, USA). Briefly, select sites on volunteers are cleaned thoroughly and allowed to air dry. Product soaked patches are adhered to the skin sites and are to be removed 48 hours later by the participant. Within 48 – 72 hours after initial patch application, trained clinicians who were evaluating any adverse reactions, including inflammation and sensitization, graded participants. Approximately two – three weeks following the first challenge, treated patches are again applied to the same sites, along with additional new sites, with 48 hour contact time and follow-up grading within the 72 hour application time frame. Subsequent Patch tests were also conducted on individual extracts to derive an approved blend of extracts of specific concentration to be utilized in a prepared topical formulation.

H.) LIPOSOME PREPARATION AND PRODUCT STABILITY

Incorporation of the select extracts into a lecithin-based liposomal (following and utilizing already established in-house protocols), leave-on topically applied product was inhibited by safety requirements and suggested DOE usage levels. This balance was achieved by extensive range testing of liposome-forming components, as well as external stabilizing compounds, skin penetrating agents and aesthetically pleasing ingredients. Trial formulations were performed following standard product development procedures using common lab equipment. Liposome development was enhanced through the use of a 110-Y Microfluidizer (Microfluidics Corp., Newton, MA, USA) to reach a desired

particle size, which would also be stable under standard stability test conditions. Successful incorporation of the liposome into an applicable external phase was determined by macroscopic stability observations (separation, haziness, discoloration and/or malodor). Particle size was determined using Microtrac's Nanotrac 150 particle size analyzer (Montgomeryville, PA, USA) and followed after exposure to both high and low temperature extremes (5°C, Ambient, 40°C and 50°C). pH stability was also followed after storage at low and high temperatures by allowing samples to equilibrate to room temperature then measured by the use of a calibrated "Basic pH Meter" (Denver Instrument, Arvada, CO, USA). Acceptable results for all of these parameters would determine successful incorporation of the extract blend into a liposomal-based usable hair growth product.

I.) MICROBIAL TESTING

Standard American Type Culture Collection (ATCC, Rockville, MD, USA) strains employed in preservative efficacy screening include *Acinetobacter baumannii*, *Aspergillus niger*, *Burkholderia cepacia*, *Candida albicans*, *Enterobacter gergoviae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. Briefly, the hair growth prototype was aseptically checked for inherent microbial contamination by being plated with microbial content agar and the number of colony forming units, for bacteria, mold and yeast tallied. The presence for objectionable organisms (Gram negative rods/cocci, *Staphylococcus aureus* and beta-hemolytic *Streptococci*) was also screened. If minimal to no growth of contaminants was measured, the prototype was then inoculated with mold (0.5 mL/100 g prototype) and bacteria/yeast inoculum (0.5 mL/100 g prototype) and tested at two, four,

seven, ten, fourteen and twenty-eight days post inoculation by spreading (10^{-2} dilution) inoculated product onto potato dextrose agar (mold and yeast) and microbial content test agar (bacteria). Self-sterilization in seven days or less is considered an adequate interval for efficacious microbial resistance (Access Business Group, LLC, Ada, MI, Internal Test Procedure, 2008).

J.) CLINICAL PROTOCOL

A general recruitment letter was sent to approximately 800 male employees at Amway Incorporated, seeking volunteer enrollment from those afflicted with androgenetic alopecia. Exclusions included those suffering from hypertension, heart disease, diabetes, thyroid diseases, metabolic disorders and current, or recently stopped users of hair growth products (topical and ingestible products). Exclusions were not made for age, degree of baldness (Hamilton-Norwood, stages 1-8), nor years of balding but these were noted. Incentives for participation included $150.00 worth of gift cards to local merchants. Amway Research & Development staff screened all individuals who expressed interest, with initial respondents numbering approximately 120. Initial screenings included visual assessment and grading of the individual's balding state, a topical head image (Canfield Visia CR, Canfield Scientific Inc. and Mirror Software, version 7.2, Canfield Imaging Systems, New Jersey, USA) and a brief questioning of medical/medicine history. Through attrition and medical issues, the total number of individuals selected for the study was 74, and this included Hamilton-Norwood scores of 1-8 and ages 18-65. The participating individuals were divided into three cells, one for each test product and the cell demographics were stratified based on age range, the Hamilton-Norwood balding score and years of experiencing a balding condition. All

participants were given, explained and signed Informed Consents, Patient Bill of Rights, and Photo Release Forms prior to study commencement.

Upon commencement of the study, beginning November, 2007, enrolled participants came to the Amway Research & Development Salon to have their entire scalp hair cut to ¼", followed by a washing with Satinique™ Gentle Daily Cleanser. After towel drying, a global, top of the scalp "cut" image was taken with the Visia CR. An Area of Interest (AOI) was identified on each individual somewhere in a balding/non-balding transition zone, with equal portions shared between the two conditions. A non-permanent dye (Clairol® Natural Instincts 36 Midnight Black) was then applied to the AOI and left on for 1.5 minutes, with minimal application to the scalp. Following the soak time, the dye was removed with wetted gauze, and from the AOI and area of 2cm X 2cm was clipped to a minimal length (approximately 1 mm length of hair remained within this zone). With a red extra fine-point permanent marker (Sanford Sharpie®, USA), 2 temporary dots were placed within the AOI using a flexible, plastic template. The center between these two reference points was measured from the left-to-right top pinna-scalp joint and from the bridge of the nose back. A "clipped" marked 30X image was captured with the ScanHair Tablet 4701 (Biomedical Electronics, Bordeaux, France) camera, captured through the Hauppauge WinTV2000 (Hauppauge, NY, USA) video capture card. The AOI was then shaved smooth with an electronic shaver and additional ScanHair "shaved" images were obtained. The red marks were re-darkened and participants were instructed not to shampoo their hair for seventy-two hours, until the follow-up visit was completed. At the follow-up appointment, the temporary marks were

relocated and re-marked as necessary. The AOI was dyed as before and washed with gauze, followed by ScanHair "72 hour" images.

At the seventy-two hour visit, participants were given their product in a six-week supply quantity, consisting of eighty-four bottles plus six extra. Each bottle contained 1.5-mL of product, equating to one application with instructions to use twice per day with a four-hour minimum leave-on period. The product was to be dispensed over the entire scalp, with special emphasis on balding/thinning areas, using a massaging technique and allowed to air dry. Ideal application times were suggested to be once per morning and once per evening. Product application occurred over a 12-week period (December 2007 –April 2008). In all, three cells were tested: Rovisomes Biotin (ROVI Cosmetics International, supplied by RITA Corporation, USA), a clinically tested hair growth prototype formula (code 586); the Access Business Group (Amway Corporation) developed botanical formula (code 883, Prototype 2); and Rogaine® Extra Strength 5% Minoxidil topical solution as a positive control (code 194).

Image analysis was performed with Image Pro Plus, version 5.1 (Media Cybernetics Inc., Silver Springs, MD, USA) and Access Business Group-developed softwares. From the "cut" global images, macro changes in hair coverage and/or pattern could be observed. Anagen-telogen ratio counts were calculated from Image Pro Plus enhanced images from Scan Hair "shaved" and "72 hour" photos, with "clipped" images serving as guides for active and non-active follicles. Growth rates (mm/72 hours) were also measured from Scan Hair "shaved" and "72 hour" photos with a calibrated 7 mm Image Pro Plus scale. AOI density measurements were calculated from "72 hour" photos, using the Access Business Group proprietary software. Density values correspond

to darkened pixel detection from the captured image and are automatically calculated as the percent area covered within the specified frame. Pre and post-treatment values (initial visit and twelve week visit, respectively) were statistically compared using the t-test. All statistical analyses were conducted using Microsoft® Office Excel, 2003 SP3 and/or StatGraphics Plus for Windows, version 2.0.

Lastly, at the conclusion of the study, participants completed self-assessment questionnaires numerically rating the efficacy of the products, attributes of the products and application procedures with results statistically compared by analysis of variance (ANOVA). Rating values were based on a nine-point scale pertaining to likes/dislikes of the hair growth product and agree/disagree statements relative to the efficacy of the products on hair growth and hair appearance and texture. Table 1 shows the rating categories and corresponding point values.

Table 1-Format of Self-Perceived Questionnaire Evaluating Product Quality & Performance

	Dislike Extremely (1)	**Dislike Very Much (2)**	**Dislike Moderately (3)**	**Dislike Somewhat (4)**	**Neither Like nor Dislike (5)**	**Like Somewhat (6)**	**Like Moderately (7)**	**Like Very Much (8)**	**Like Extremely (9)**
Overall Liking Aroma									
	Disagree Very Strongly (1)	Disagree Strongly (2)	Disagree Moderately (3)	Disagree (4)	Neither Agree nor Disagree (5)	Agree (6)	Agree Moderately (7)	Agree Strongly (8)	Agree Very Strongly (9)
Easy Dispension									
Quick Absorption									
Enhanced Natural Hair									
Improved Hair Appearance									
Improved Texture									
Improved Density									
Concept was Intriguing									

CHAPTER V- RESULTS

A.) SELECTION OF BOTANICAL EXTRACTS

Composite botanical extract efficacy was calculated as the sum of trial performances for each bioassay, with multiple trials being run for each extract in several assays. Values for scoring (Table 2) were based on the effect of the extract to up or down regulate each biomarker, relative to a media-only control for VEGF and KGF, and an enzyme/substrate positive control for proteasome function ran in conjunction with each trial.

Table 2-Extract Scoring for in-vitro Assays

Bioassay	% Control Range	Extract Score
VEGF/KGF	>300	30
	300-276	25
	275-199	20
	200-176	15
	175-151	10
	150-126	5
	125-76	0
	75-51	-5
	50-26	-10
	<25	-15
Proteasome	>169	-20
	169-150	-15
	149-130	-10
	129-110	-5
	109-90	0
	89-70	5
	69-50	10
	49-30	15
	<30	20

Several of the extracts were tested multiple times (n values in Table 3) to understand response variability in the cell-culture systems (VEGF and KGF) and to set the range of expression for scoring purposes. IL-1α was only run for the DOE portion of the research due to timing issues and controversy of the biomarker in HF regulation. Extracts and composite scores are listed in Table 3. Of the forty-nine listed extracts, seven were selected for potential incorporation into a usable product (Boswin 30,

Lichochalcone, Phycojuvenine, Saw Palmetto, Shiso, Teavigo Phospholipids and Green Rooibos) based on having the overall highest composite scores. From the selected list of seven extracts, two were eliminated, one based on patent protection for using the extract, or a derivative of it, in another hair growth claiming product (Teavigo Phospholipids: epigallocatechin gallate in US Patent 106263A1) and one based on comparable performance (i.e. Phycojuvenine compared to Saw Palmetto) to the five remaining selected extracts. The final ingredients selected for optimization, and possible synergistic effects, were Lichochalcone, Saw Palmetto, Shiso, Green Rooibos and Boswin 30.

Table 3- Extract Scores in Bioassays with extracts in bold selected for further consideration in formula development

#	Extract	VEGF	KGF	Proteasome	Composite Score	n
1	Amentoflavone	10	-5	20	25	14
2	Apple Extract (Appol)	-20	-5	15	-10	14
3	Applephenon SH	5	0	5	10	16
4	Astaxanthin	10	30	-10	30	23
5	Astilibin	-5	-	10	0	14
6	Avocutine	5	5	-10	0	17
7	Bamboo (R1563)	30	0	-10	20	17
8	Bamboo AOB (R1558)	-10	30	15	35	14
9	Bamboo EOB-CO2 (R1555)	10	15	0	25	20
10	Bamboo EOB-PO1 (R1560)	-5	20	10	25	11
11	Bamboo EOB-SO1 (R1557)	0	15	-5	10	14
12	Bamboo EOB-SO2 (R1556)	0	30	-5	25	11
13	Bamboo Essence 1 (ACCL)	20	20	-5	35	14
14	Bamboo Essence 2 (ACCL)	-20	35	-15	0	14
15	Bamboo EZR-2002 (R1559)	-10	35	10	35	11
16	Bamboo Stachyse (R1562)	0	15	-5	10	17
17	Boswellia serrata	-10	-10	25	5	14
18	Boswellin forte	5	0	30	35	20
19	**Boswin 30**	**30**	**15**	**25**	**70**	**17**
20	Boswin 30 (5-loxin)	-5	-10	20	5	22
21	Centella asiatica extracts	0	-15	-5	-20	16
22	Cocoa Extract	-5	-10	-10	-25	16
23	CoQ10 TPM	15	0	-10	5	17
24	Cosmoperine	-20	-30	-10	-60	14
25	Ellagic Acid	-20	-15	10	-25	14
26	GFS 75%	15	20	0	35	20
27	Grape Seed Oil	-10	-15	-10	-35	16
28	Gravinol-S	-25	20	15	10	16
29	Green Coffee Antioxidant	-5	30	-5	20	14
30	Hops Oleoresin	-10	-10	0	-20	14
31	Isoginketin 74%	0	-10	20	10	15
32	Kudzu Extract	-5	-5	-30	-40	14
33	**Lichochalcone**	**40**	**40**	**40**	**120**	**25**
34	Luteolin	-25	-10	0	-35	14
35	**Phycojuvenine**	**30**	**30**	**-5**	**55**	**17**
36	Phytavail Zn	-5	-5	-5	-15	16
37	Red Clover Special	-5	-20	-25	-50	8
38	Rogaine®	0	-10	-10	-20	14
39	Salvia	-5	0	-15	-20	26
40	**Saw Palmetto (Euromed)**	**30**	**35**	**-5**	**60**	**19**
41	Saw Palmetto	5	45	-5	45	20
42	**Shiso**	5	50	-10	45	31
43	Soy Extract	15	15	-10	20	15
44	Teavigo	5	-10	20	15	16
45	**Teavigo Phospholipids**	**25**	**15**	**10**	**50**	**14**
46	**Green Rooibos**	**0**	**50**	**-5**	**45**	**22**
47	Vitagen	-5	-5	-10	-20	14
48	Vital ET	-5	0	5	0	8
49	Ximenynic Acid	-15	15	-10	-40	20

B.) DOE ANALYSIS

DOE 1 (Table 4) and DOE 2 (Table 5) analyses showed at least one of the extracts had a significant influence (ANOVA) in all four bioassays (Table 6), and response data did fit the regression models in all cases.

Table 4- DOE 1 Layout

Run	Lichochalcone	Boswin 30	Saw Palmetto	Shiso	Green Rooibos
1	0	0.5	1	0	0
2	0	0	1	0	1
3	0.25	0.75	0.25	0.25	0.75
4	0.5	1	1	1	0
5	1	1	0	0	0
6	0	0	1	0	1
7	0	0.5	0	1	0
8	1	0	0.5	0	1
9	1	0	1	0.5	0
10	0	0	0.5	1	1
11	0.5	0.5	0.5	0.5	0.5
12	0.75	0.25	0.25	0.75	0.75
13	1	1	1	0	1
14	1	0	0	1	0
15	0.5	0	0	0	0
16	0.75	0.75	0.75	0.25	0.25
17	0.5	0	1	1	1
18	0.25	0.25	0.75	0.75	0.75
19	1	0	0	1	0
20	0	0	0	0	0
21	0.75	0.75	0.25	0.25	0.75
22	0.75	0.25	0.25	0.25	0.75
23	0	1	0.5	0	1
24	0	1	1	0	0.5
25	1	0	0	0.5	1
26	0.5	1	0	1	1
27	0.25	0.75	0.25	0.75	0.25
28	0	0	1	1	0
29	0.5	0.5	0.5	0.5	0.5
30	0.75	0.25	0.75	0.25	0.25
31	0	0.5	1	0	0
32	0	0	1	1	0
33	0.5	1	0	1	0
34	0.25	0.75	0.75	0.75	0.75
35	0.75	0.75	0.75	0.75	0.25
36	0	0	0	1	1
37	1	0	1	0	0.5
38	1	0.5	0	0	1
39	0	1	0	0	0
40	1	1	0	1	0.5
41	0	1	1	1	1
42	1	1	1	1	1
43	0	0.5	0	0	1
44	0	1	0	0	0
45	0	0	0	1	0
46	0	0	1	0	0
47	0	0	0	0	1
48	1	0	0	0	0
49	0	0	0	0	0
50	0	0	0	0	0

Table 5- DOE 2 Layout

Run	Lichochalcone	Saw Palmetto	Shiso	Green Rooibos
1	0.1	0.5	0	0
2	0.1	0.5	25	0
3	0.1	12.75	0	0
4	0.1	25	25	0
5	0.1	25	0	12.5
6	0.1	0.5	0	25
7	0.1	0.5	0	25
8	0.1	0.5	25	25
9	0.1	12.75	25	25
10	0.1	25	12.5	25
11	0.2	6.625	18.75	6.25
12	0.2	18.875	6.25	6.25
13	0.2	6.625	18.75	18.75
14	0.2	18.875	18.75	18.75
15	0.3	25	0	0
16	0.3	12.75	12.5	12.5
17	0.3	12.75	12.5	12.5
18	0.3	25	25	25
19	0.4	18.875	18.75	6.25
20	0.4	18.875	6.25	6.25
21	0.4	6.625	6.25	18.75
22	0.4	18.875	18.75	18.75
23	0.5	0.5	25	0
24	0.5	0.5	0	0
25	0.5	0.5	25	0
26	0.5	25	12.5	0
27	0.5	25	25	0
28	0.5	25	25	0
29	0.5	25	0	12.5
30	0.5	25	25	12.5
31	0.5	0.5	0	25
32	0.5	0.5	25	25
33	0.5	25	0	25
34	0.5	25	12.5	25
35	2.875	6.625	18.75	6.25
36	2.875	18.875	6.25	6.25
37	2.875	18.875	6.25	18.75
38	2.875	18.875	18.75	18.75
39	5.25	25	0	0
40	5.25	0.5	12.5	12.5
41	5.25	12.75	12.5	12.5
42	5.25	12.75	12.5	12.5
43	5.25	25	25	25
44	7.625	18.875	18.75	6.25
45	7.625	18.875	6.25	6.25
46	7.625	6.625	6.25	18.75
47	7.625	18.875	18.75	18.75
48	10	0.5	0	0
49	10	0.5	25	0
50	10	25	12.5	0
51	10	25	25	12.5
52	10	0.5	25	25
53	10	25	0	25
54	10	25	0	25

Multiple linear regression analysis indicated that several of the extracts had significant impacts on either up or down regulation (% control) of the specific biomarkers.

Table 6- Results of DOE Analyses 1 & 2 (L= Lichochalcone, GR= Green Rooibos, P=Saw Palmetto, S= Shiso)

DOE 1					
Bioassay	**df**	**R^2 Value**	**p value**	**n**	**Regression Models (All contributing extracts listed, p < 0.05)**
VEGF	3	0.59	< 0.0001	50	5.493 – 3.823 (Ex L) + 2.942(Ex GR)^2 – 1.251 (Ex. P * Ex S)
KGF	2	0.61	< 0.0001	50	17.433 + 1.667(Ex L) – 1.886(Ex L)^2
Proteasome	6	0.80	< 0.0001	50	25361 – 3236(Ex L) – 649(Boswin 30) – 771(Ex L * Boswin 30) + 652(Ex L * Ex GR) + 771(Boswin 30 * Ex S) – 660(Ex S * Ex GR)
IL-1α*	2	0.16	0.02	50	3.877 + 0.79(Boswin 30) – 0.076(Ex P * Ex GR)
DOE 2					
VEGF*	3	0.64	< 0.0001	54	4.756 – 1.321(Ex L) – 1.513(Ex P) – 1.416(Ex L * Ex P)
KGF	3	0.91	< 0.0001	54	36.78404 –28.36144(Ex L) – 5.70125(Ex P) - 4.57404(Ex GR)^2
Proteasome*	6	0.98	< 0.0001	54	0.162 + 0.044(Ex L) – 0.030(Ex L)^2 + 0.002(Ex L * Ex GR) + 0.002(Ex L * Ex P) + 0.002(Ex L * Ex S) + 0. 002(Ex GR * Ex P)
IL-1α*	4	0.70	< 0.0001	54	5.271 + 0.202(Ex L) + 0.213(Ex P) – 0.242(Ex L)^2 – 0.091(Ex L * Ex P)

For VEGF, Lichochalcone was a down-regulator in both DOE studies. Saw Palmetto, either in conjunction with Shiso (DOE 1) or Lichochalcone (DOE 2), also had a similar effect. Higher concentrations of Green Rooibos negatively affected VEGF production. KGF expression (pg/mL) had relatively high baselines in both DOEs, but Lichochalcone had contradictory effects. In DOE 1, a dose-dependent effect was observed for Lichochalcone, with higher concentrations leading to suppression of VEGF production. Lichochalcone, on the other hand, was predominantly suppressive of KGF in DOE 2, as was Saw Palmetto and higher concentrations of Green Rooibos.

Proteasome function was definitely suppressed by Lichochalcone and Boswin 30, both separately and in combination, in DOE 1. Interestingly, when Lichochalcone or Boswin 30 were combined with Green Rooibos and Shiso, respectively, proteasome function was enhanced, while Green Rooibos and Shiso in combination down-regulated

* Data required transformation prior to analysis. For IL-1α, DOE 1, e raised to the regression model listed; for DOE 2, the transformation involved the sum of –3.194 + e raised to the model sum. For VEGF in DOE 2, the VEGF sum depicted was squared.

proteasome activity, possibly indicative of antagonistic effects of these compounds. In DOE 2, a higher concentration of Lichochalcone led to a reduction of proteasome function, while all other significant combinations tended to slightly up-regulate the proteasome. Lichochalcone, Boswin 30 and Saw Palmetto enhanced IL-1α expression. Saw Palmetto in combination with Green Rooibos suppressed IL-1α expression (DOE 1), as did Saw Palmetto with Lichochalcone (DOE 2). Since Boswin 30 demonstrated proteasome inhibition, but was less effective than Lichochalcone, as well as being a slight up regulator of IL-1α, it was dropped from consideration as a potential hair growth promoting extract.

When the DOE optimization study was conducted, a total of seventeen combinations of extracts were screened, with ranges of each extract derived from DOE 1 and DOE 2. From these studies, and corresponding analysis, three combinations of extracts were determined to be suitable for product incorporation, whereas only one combination had a positive influence in more than one of the bioassays. The final optimal blend of extracts consisted of Lichochalcone at 2.5 μg/mL, Green Rooibos at 12.5 μg/mL, Saw Palmetto at 4.5 μg/mL and Shiso at 12.5 μg/mL. From the elicited dose cellular responses, it was decided to treat these as tenfold concentrations in an actual use product, thereby resulting in levels of 0.25% for Lichochalcone, 1.25% for Extracts Green Rooibos and Shiso and 0.45% for Saw Palmetto.

C.) INGREDIENT FINALIZATION

Due to time constraints, a maximally concentrated botanical prototype formulation (Prototype 1) was submitted for Repeat Insult Patch Testing to rule out severe irritancy issues and possible allergic sensitization occurrences in humans.

Prototype 1 consisted of 1% each of extracts Green Rooibos, Saw Palmetto and Shiso and 0.20% of Lichochalcone and 5% each of three skin penetration enhancers. Of the 109 subjects challenged and re-challenged with Prototype 1, one individual definitively showed a sensitization response, along with five other potential participants. This formulation also caused significant irritation. As a result, Prototype 1 was not approved for further testing, in accordance with inherent corporate standards. Mild irritation responses are acceptable for research continuation, but when severe irritation arises, coupled with potentially widespread sensitization, re-formulation is mandated. Subsequent submissions to TKL Research included: the vehicle of Prototype 1 only; individual extracts along with the vehicle; reduced levels of both individual extracts and penetration enhancers; and ultimately, diluted extracts only, tested individually. Through the process of elimination, it was determined that the excessively high concentrations of the skin penetration enhancers, in combination with the maximal concentration of the botanical extracts, led to the sensitization and irritancy issues. The final approved usage levels of each extract were: Lichochalcone at 0.25%, Green Rooibos at 1.0%, Saw Palmetto at 0.45%, and Shiso at 1.25%. The skin penetration enhancers were capped at 0.4% usage levels.

D.) LIPOSOME PREPARATION & PRODUCT STABILITY

The last hurdles of extract incorporation into a testable hair growth product were assembling extract-containing liposomes into a final formulation and achieving product stability in terms of liposome size, pH and aesthetic character. A final formula was developed with tolerable levels of hydrophobic extracts (Lichochalcone and Saw Palmetto) incorporated into the lipid membrane of the liposome, while the hydrophilic

extracts (Green Rooibos and Shiso) were placed within the aqueous core of the liposome. The remaining portions of each extract, up to their allowable levels, were placed within either the water external phase or oil external phase, so as to achieve maximal concentration. The final product, Prototype 2, is shown in Table 7 with select ingredient ranges listed due to the proprietary and patent pending technology tested. Prototype 2 was successfully prepared in the lab, with the liposomal phase undergoing three passes in the microfluidizer to achieve the desired particle size of 100 nm – 300 nm.

Table 7- Formulation of Prototype 2

Phase A (Water External Phase)	
Chemical Name	**Concentration**
Purified water	*qs*
Diethylene glycol monoethyl ether	0.1 – 0.5%
Dimethyl Isosorbide	0.1 – 0.5%
Shiso	0.6250%
Green Rooibos	0.9000%
1,2,3-Propanetroil	1.0 – 4.0%
End Phase A	
Phase B (Oil External Phase)	
Polyoxyethylene Oleyl Ether	0.1 – 2.0%
1,3-Butanediol	0.1 – 1.0%
1,3-Dioxolan-2-one, 4-methyl	0.1 – 1.0%
Lichochalcone	0.2125%
Saw Palmetto	0.2250%
Denatured Ethanol	10 - 15%
End Phase B	
Phase C (Liposome Phase)	
1,3-Butanediol	5.0 – 8.0%
Phosphatidlycholine	4.0 – 7.0%
N-Oleoyl Phytosphingosine	0.1 – 0.2%
Beta-Sitosterol	0.01 – 0.5%
Mixed Tocopherols	0.01 – 0.1%
Saw Palmetto	0.2250%
Lichochalcone	0.0375%
Shiso	0.6250%
Green Rooibos	0.1000%
Purified water	30 - 40%
End Phase C	
Phenoxyethanol, Methyl-, Propyl- & Ethylparaben	0.5 - 1.0%
Arginine	0.01 – 0.02%

A multi-environment stability was then conducted with the prototype stored in glass jars. At subsequent evaluation points, the prototype's pH and aesthetic properties (Table 8) were evaluated, as was liposome particle size (Figure 4) and these were compared to specification range and initial production values. The initial production pH was below the specification range, most likely due to inadequate pH adjustment during manufacturing. For the 5°C condition, Prototype 2 retained all acceptable attributes throughout the test. Ambient stored samples exhibited a slight alteration in odor but were satisfactory for color and appearance attributes. At both elevated temperature conditions, noticeable discoloration occurred, as did a very slight thickening of the product. The fragrance lost most of its tea-like character, which gave way to an oil-based solvent scent.

Table 8- Stability Profile of Prototype 2 Formula

	pH	**Aesthetic Attributes: color, appearance & odor[1]**
Specification	**5.7-7.5**	**Acceptable**
Initial	5.5[2]	Amber, translucent liquid w/ ethanolic, tea-like fragrance
1 Month @ 5°C	5.6	Acceptable
2 Months @ 5°C	5.5	Acceptable
3 Months @ 5°C	5.5	Acceptable
1 Month @ Ambient	5.6	Amber w/ more medicinal odor
2 Months @ Ambient	5.4	No change
3 Months @ Ambient	5.4	No change
1 Month @ 40°C	5.5	Amber, more viscous & more solvent notes
2 Months @ 40°C	5.3	No change
3 Months @ 40°C	5.2	Light, dirty brown color; more translucent w/ more solvent character
1 Month @ 50°C	5.5	Light, dirty brown color; more viscous w/ solvent character

[1] The formulator evaluates aesthetic attributes with comparisons made to the 5°C samples.
[2] Immediately after production the measured pH was out of the specification range of 5.7 – 7.5. This was due to an inadequate amount of added arginine to elevate the pH. All subsequent values were then out of specification. The biggest drop in pH (3 months at 40°C) to 5.22 is not abnormal for this product type. An adequate amount of arginine would be needed to compensate for this drop to keep the product within the specification range after elevated temperature storage.

For the ideal particle size range (Figure 4), there was no significant difference between the initial sample and all stability samples (ANOVA, $p = 0.34$). In fact, for all particle sizes (data not shown) measured (0.95 nm – 6540 nm, n =52), there was no significant difference between any of the eleven samples (ANOVA, $p = 1.0$).

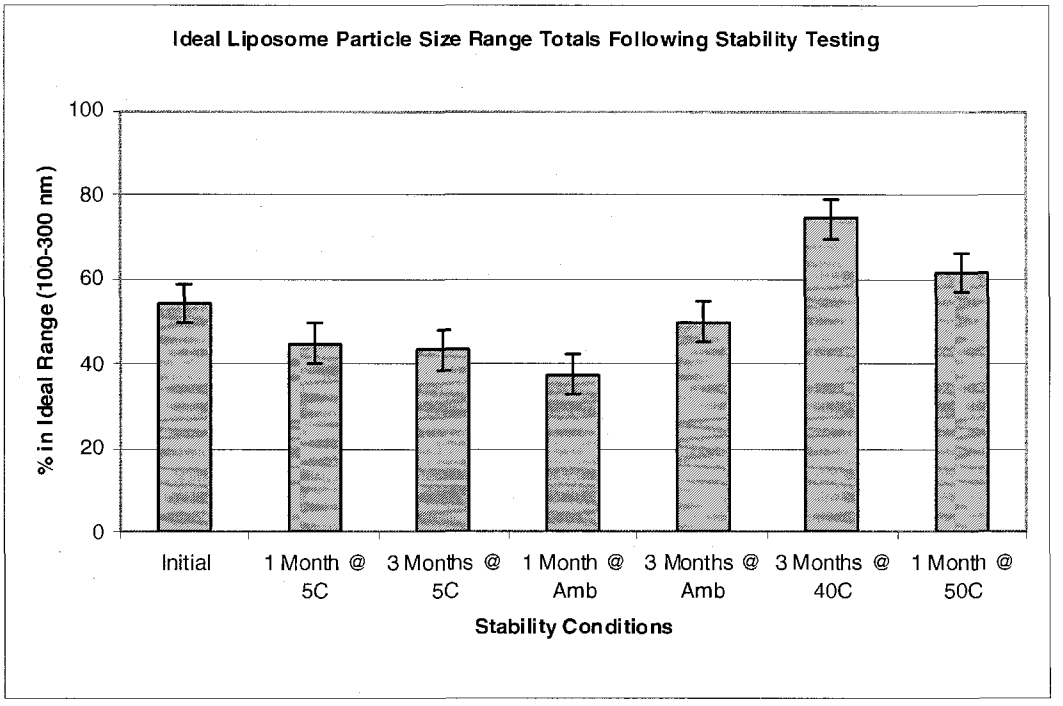


Figure 4- Liposome Particles in the Ideal Size Range for Prototype Stability Samples (+/- SE)

Prototype 2 was intrinsically clean with less than one hundred colony- forming units of aerobic bacteria, yeast and mold isolated during plate counts. For the self-sterilization portion of the microbial testing, performance was robust for eliminating bacteria, yeast and mold. With bacteria and yeast inoculation, product self-sterilization occurred in less than two days, while with mold, self-sterilization took less than four days. All results indicate Prototype 2 is a well-preserved formulation and meets industry standards for microbial safety.

F.) CLINICAL STUDY

Cell demographics are detailed in Tables 9 - 11.

Table 9 - Product 586 User Demographics with users in bold eventually dropping from the study. Adjusted values include only those individuals who remained in the study for the full 12 weeks.

Cell #1- Rovisomes			
Number	Age Range	N-H Balding Score	Years Balding
101	**39-49**	**8**	**20**
102	29-39	3	5
103	49-59	7	20
104	**18-29**	**4**	**2**
105	39-49	5	5
106	29-39	6	10
107	39-49	4	10
108	39-49	7	18
109	39-49	7	15
110	49-59	6	5
111	39-49	5	23
112	29-39	7	10
113	39-49	8	10
114	49-59	7	35
115	29-39	3	20
116	29-39	8	10
117	18-29	7	9
118	29-39	8	10
119	39-49	3	1
120	49-59	6	20
121	29-39	5	2
122	39-49	8	15
123	29-39	2	8
124	29-39	1	1
125	39-49	8	10
126	29-39	4	3
Mean		5.72	11.8
Adjusted		5.63	11.46
Median	39-49	6	10

Table 10- Product 883 User Demographics with users in bold eventually dropping from the study. Adjusted values include only those individuals who remained in the study for the full 12 weeks.

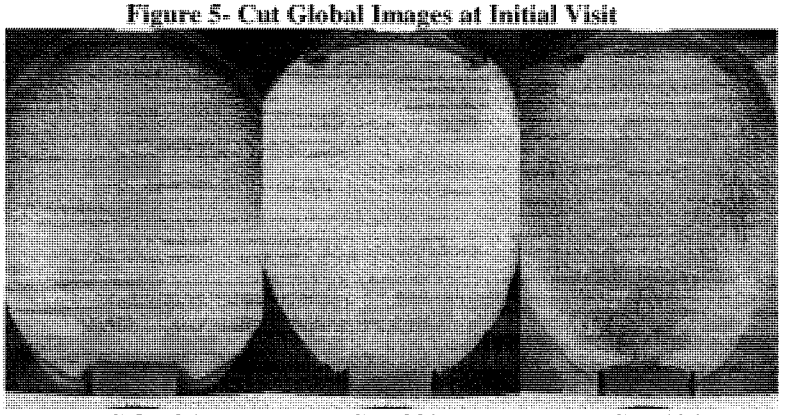
Cell #2- Prototype 2			
Number	Age Range	N-H Balding Score	Years Balding
201	59-69	4	10
202	39-49	5	20
203	49-59	7	10
204	39-49	2	5
205	39-49	4	2
206	49-59	3	15
207	18-29	8	10
208	39-49	6	20
209	59-69	7	20
210	39-49	7	10
211	39-49	7	15
212	39-49	8	18
213	49-59	8	20
214	39-49	8	25
215	49-59	6	6
216	39-49	4	10
217	49-59	6	7
218	39-49	5	7
219	49-59	7	5
220	39-49	7	15
221	29-39	5	3
222	**39-49**	**8**	**20**
223	29-39	6	4
224	29-39	3	5
Mean		5.875	11.75
Adjusted		5.78	11.39
Median	39-49	6	10

Table 11- Product 194 User Demographics with users in bold eventually dropping from the study. Adjusted values include only those individuals who remained in the study for the full 12 weeks

Cell #3- Rogaine® 5% Minoxidil			
Number	Age Range	N-H Balding Score	Years Balding
301	39-49	6	18
302	39-49	8	25
303	49-59	8	20
304	49-59	7	22
305	49-59	7	20
306	49-59	4	3
307	49-59	4	5
308	49-59	6	19
309	**49-59**	**7**	**10**
310	49-59	5	10
311	49-59	8	20
312	29-39	6	3
313	39-49	2	5
314	39-49	4	17
315	49-59	5	12
316	49-59	8	17
317	39-49	3	10
318	29-39	7	8
319	39-49	7	15
320	49-59	6	5
321	**49-59**	**7**	**10**
322	29-39	7	12
323	39-49	3	10
324	29-39	7	16
Mean		5.87	13.13
Adjusted		5.82	13.27
Median	49-59	6.5	12

Representative global "Cut", AOI "Clipped" and "Shaved" and "72 Hour" follow up images from all three cells at the initial visit are depicted in Figures 5 – 8.

Figure 5- Cut Global Images at Initial Visit

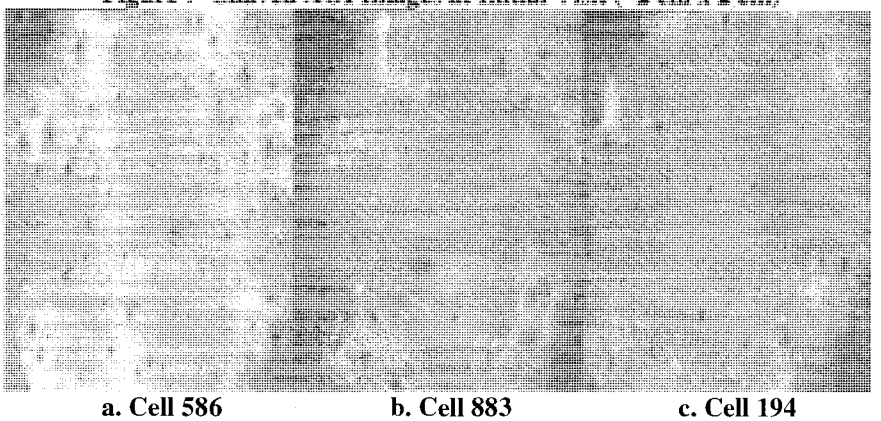


a. Cell 586 **b. Cell 883** **c. Cell 194**

Figure 6- Clipped AOI Images at Initial Visit (~2 cm x 2 cm)

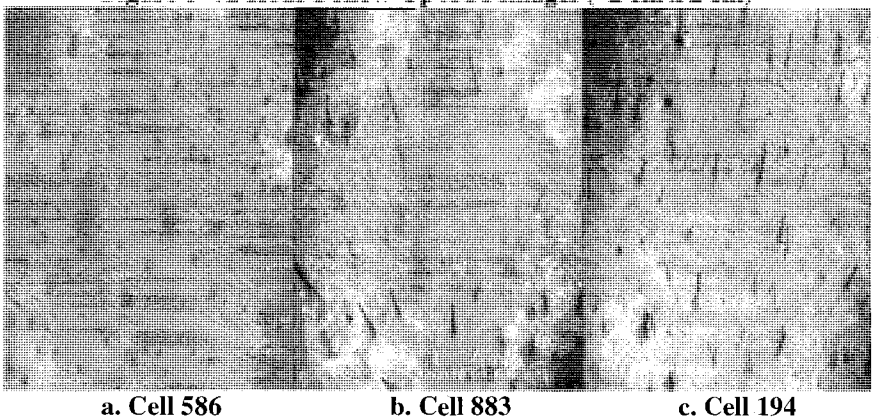
a. Cell 586 **b. Cell 883** **c. Cell 194**

Figure 7- Shaved AOI Images at Initial Visit (~2 cm x 2 cm)



a. Cell 586 **b. Cell 883** **c. Cell 194**

Figure 8- 72 Hour Follow-Up AOI Images (~2 cm x 2 cm)



a. Cell 586 **b. Cell 883** **c. Cell 194**

Tables 12-14 represent the change in global hair density per individual in each cell, with dropped participants omitted.

Table 12- Change in Density over 12 weeks for Rovisomes

Number	Pre-Treatment Density	Post-Treatment Density	% Change
102	2.46	2.75	11.8%
103	1.14	2.27	99.1%
105	3.01	2.95	-2.0%
106	2.58	2.23	-13.6%
107	3.60	6.14	70.6%
108	3.22	3.03	-5.9%
109	2.27	4.62	103.5%
110	1.86	3.77	102.7%
111	2.56	2.83	10.5%
112	2.91	2.09	-28.2%
113	1.48	2.00	35.1%
114	1.95	1.57	-19.5%
115	2.84	1.88	-33.8%
116	2.96	1.62	-45.3%
117	2.07	2.90	40.1%
118	3.89	4.06	4.4%
119	2.77	3.31	19.5%
120	1.81	2.04	12.7%
121	3.00	3.80	26.7%
122	1.86	1.90	2.2%
123	1.54	3.29	113.6%
124	3.71	6.58	77.4%
125	1.37	2.06	50.4%
126	2.31	5.69	146.3%

Table 13- Change in Density over 12 weeks for Prototype 2

Number	Pre-Treatment Density	Post-Treatment Density	% Change
201	1.97	5.22	165.0%
202	3.01	2.87	-4.7%
203	4.85	5.74	18.4%
204	6.51	7.16	10.0%
205	2.70	4.08	51.1%
206	3.38	3.88	14.8%
207	5.02	7.27	44.8%
208	4.40	4.62	5.0%
209	2.11	3.40	61.1%
210	2.79	3.25	16.5%
211	1.67	1.63	-2.4%
212	3.23	3.57	10.5%
213	0.71	0.72	1.4%
214	1.21	1.62	33.9%
215	1.44	1.62	12.5%
216	4.12	4.44	7.8%
217	2.53	2.60	2.8%
218	2.39	2.78	16.3%
219	2.96	3.74	26.4%
220	1.78	2.20	23.6%
221	2.04	2.15	5.4%
223	2.75	3.59	30.5%
224	5.01	5.12	2.2%

Table 14- Change in Density over 12 weeks for Rogaine®

Number	Pre-Treatment Density	Post-Treatment Density	% Change
301	1.66	1.97	18.7%
302	3.78	3.77	-0.3%
303	2.50	3.76	50.4%
304	2.41	2.64	9.5%
305	1.53	1.71	11.8%
306	1.85	3.48	88.1%
307	2.70	2.89	7.0%
308	1.71	1.66	-2.9%
310	2.48	2.76	11.3%
311	2.21	3.28	48.4%
312	3.28	5.50	67.7%
313	6.03	7.53	24.9%
314	1.22	2.33	91.0%
315	3.11	3.26	4.8%
316	1.34	1.69	26.1%
317	5.16	6.19	20.0%
318	2.68	3.72	38.8%
319	2.84	2.82	-0.7%
320	1.40	1.43	2.1%
322	1.95	2.36	21.0%
323	3.96	3.99	0.8%
324	1.93	2.33	20.7%

The average change in density for the Rovisomes material was 32.4%, while that for Prototype 2 and Rogaine® were 24% and 25.4%, respectively. Pre- and post-treatment comparisons (t-test) revealed usage of all three compounds caused significant up-regulation in hair density (Rovisomes, $p = 0.01$; Prototype 2, $p = 0.0008$; and Rogaine®, $p = 0.0002$). When the percent changes were statistically compared (ANOVA) between the compounds, no significant difference existed (Table 15).

Table 15-ANOVA of Percent Change in Hair Growth Density over 12 weeks for all 3 Cells (Values transformed [$\log_{10}$(% change^2)] to correct for non-normal distributions).

Source	Sum of Squares	df	Mean Square	F-ratio	P-value
Between Groups	6.45	2	3.23	2.18	0.12
Within Groups	101.92	69	1.48		
Total	108.37				

From the Anagen-Telogen ratio counts, the calculated percentage of hair follicles in the Anagen stage are broken down by cell and are displayed in Tables 16-18.

Table 16- Change in Anagen Hair Follicles over 12 weeks for Rovisomes

Number	Pre-Treatment Anagen	Post-Treatment Anagen	% Change
102	38.74	49.26	27.16%
103	55.19	59.20	7.27%
105	37.80	60.88	61.06%
106	24.01	38.28	59.43%
107	38.28	75.59	97.47%
108	42.39	61.83	45.86%
109	67.51	88.61	31.25%
110	40.53	74.07	82.75%
111	63.54	64.91	2.16%
112	28.57	39.38	37.84%
113	62.14	45.13	-27.37%
114	57.31	69.95	22.06%
115	50.89	45.54	-10.51%
116	77.18	70.90	-8.14%
117	60.59	57.97	-4.32%
118	70.53	67.08	-4.89%
119	56.37	49.39	-12.38%
120	52.65	71.57	35.94%
121	55.04	56.11	1.94%
122	56.20	48.00	-14.59%
123	7.88	27.33	246.83%
124	56.00	82.46	47.25%
125	49.74	31.48	-36.71%
126	63.08	84.47	33.91%

Table 17- Change in Anagen Hair Follicles over 12 weeks for Prototype 2

Number	Pre-Treatment Anagen	Post-Treatment Anagen	% Change
201	43.29	84.10	94.27%
202	72.70	41.03	-43.56%
203	69.32	87.26	25.88%
204	81.98	93.50	14.03%
205	45.02	69.39	54.13%
206	58.14	79.43	36.62%
207	71.61	89.50	24.98%
208	65.28	80.91	23.94%
209	27.69	54.23	95.85%
210	43.19	49.79	15.28%
211	50.10	66.88	33.49%
212	34.50	59.92	73.68%
213	19.49	37.82	94.05%
214	32.82	43.52	32.60%
215	16.32	30.16	84.80%
216	62.46	68.82	10.18%
217	57.21	89.91	57.16%
218	50.81	75.55	48.69%
219	66.59	77.03	15.68%
220	30.16	37.20	23.34%
221	45.16	43.41	-3.88%
223	59.59	69.36	16.40%
224	78.71	85.60	8.78%

Table 18- Change in Anagen Hair Follicles over 12 weeks for Rogaine®

Number	Pre-Treatment Anagen	Post-Treatment Anagen	% Change
301	47.55	64.35	35.33%
302	52.53	57.10	8.70%
303	35.37	69.80	97.34%
304	41.44	53.71	29.61%
305	30.52	34.37	12.61%
306	44.42	71.12	60.11%
307	50.05	80.49	60.82%
308	45.73	64.82	41.75%
310	50.59	69.61	37.60%
311	57.68	90.71	57.26%
312	71.35	88.00	23.34%
313	76.26	88.59	16.17%
314	34.46	41.53	20.52%
315	51.72	71.18	37.63%
316	55.53	34.40	-38.05%
317	77.22	91.20	18.10%
318	46.78	64.51	37.90%
319	48.19	54.72	13.55%
320	29.06	18.83	-35.20%
322	36.29	44.69	23.15%
323	71.20	81.49	14.45%
324	34.06	41.53	21.93%

All three-test products exhibited a positive increase in the number of anagen hair follicles over the twelve-week period. Rovisomes yielded an average increase of 30.05%, while that for Prototype 2 was 36.37% and for Rogaine®, 27.03%. Statistically, all increases in the number of anagen hair follicles for each product were significant (Rovisomes, $p = 0.01$; Prototype 2, $p = 0.00004$; and Rogaine®, $p = 0.0001$). Comparison between the three cells, showed no significant difference (Kruskal-Wallis) in the ability to promote anagen follicle induction ($p = 0.54$). Data transformation in the form of $\log_{10}(x^2)$ was required prior to analysis to achieve a normal distribution.

The last measure of performance determined from the AOI Scan Hair images was growth rate. Before and after treatment growth rate values are displayed in Tables 19-21.

Table 19- Change in Growth Rate over 12 Weeks for Rovisomes

Number	Pre-Treatment Growth Rate (mm/72hrs)	Post-Treatment Growth Rate (mm/72hrs)	% Change
102	0.68	0.57	-19.30%
103	0.46	0.50	8.00%
105	0.45	0.38	-18.42%
106	0.40	0.33	-21.21%
107	0.63	0.54	-16.67%
108	0.41	0.44	6.82%
109	0.58	0.64	9.38%
110	0.48	0.60	20.00%
111	0.51	0.53	3.77%
112	0.56	0.61	8.20%
113	0.48	0.56	14.29%
114	0.57	0.36	-58.33%
115	0.59	0.52	-13.46%
116	0.57	0.37	-54.05%
117	0.72	0.72	0.00%
118	0.59	0.63	6.35%
119	0.62	0.78	20.51%
120	0.47	0.49	4.08%
121	0.65	0.61	-6.56%
122	0.59	0.49	-20.41%
123	0.41	0.59	30.51%
124	0.54	0.60	10.00%
125	0.37	0.44	15.91%
126	0.71	0.65	-9.23%

Table 20- Change in Growth Rate over 12 Weeks for Prototype 2

Number	Pre-Treatment Growth Rate (mm/72hrs)	Post-Treatment Growth Rate (mm/72hrs)	% Change
201	0.52	0.65	25.00%
202	0.70	0.73	4.29%
203	0.63	0.79	25.40%
204	0.60	0.67	11.67%
205	0.55	0.78	41.82%
206	0.50	0.55	10.00%
207	0.82	0.95	15.85%
208	0.62	0.77	24.19%
209	0.51	0.62	21.57%
210	0.54	0.53	-1.85%
211	0.55	0.35	-36.36%
212	0.55	0.67	21.82%
213	0.38	0.42	10.53%
214	0.45	0.45	0.00%
215	0.64	0.67	4.69%
216	0.74	0.56	-24.32%
217	0.54	0.66	22.22%
218	0.43	0.59	37.21%
219	0.50	0.60	20.00%
220	0.49	0.63	28.57%
221	0.47	0.55	17.02%
223	0.63	0.65	3.17%
224	0.57	0.56	-1.75%

Table 21- Change in Growth Rate over 12 Weeks for Rogaine®

Number	Pre-Treatment Growth Rate (mm/72hrs)	Post-Treatment Growth Rate (mm/72hrs)	% Change
301	0.49	0.42	-14.29%
302	0.73	0.55	-24.66%
303	0.64	0.66	3.13%
304	0.57	0.57	0.00%
305	0.41	0.43	4.88%
306	0.48	0.56	16.67%
307	0.48	0.63	31.25%
308	0.39	0.40	2.56%
310	0.50	0.42	-16.00%
311	0.67	0.72	7.46%
312	0.63	0.81	28.57%
313	0.66	0.67	1.52%
314	0.49	0.63	28.57%
315	0.53	0.53	0.00%
316	0.67	0.67	0.00%
317	0.56	0.53	-5.36%
318	0.47	0.63	34.04%
319	0.57	0.59	3.51%
320	0.48	0.44	-8.33%
322	0.48	0.46	-4.17%
323	0.49	0.53	8.16%
324	0.53	0.37	-30.19%

For the Rovisomes material, the average change in growth rate was –3.33% over the twelve-week treatment period. Both Prototype 2 and Rogaine® had positive increases in growth rate over the same time span, 12.21% and 3.06%, respectively. The change in growth rate for all cells was not significant (Rovisomes, $p = 0.90$; Prototype 2, $p = 0.07$; Rogaine®, $p = 0.67$) at the 95% confidence level. When the change in growth rate of all three-test products was compared (ANOVA, Table 22), a significant difference was found to exist between Rovisomes and Prototype 2 ($p = 0.016$).

Table 22- ANOVA of Percent Change in Growth Rate over 12 Weeks for all 3 Cells

Source	Sum of Squares	df	Mean Square	F-ratio	P-value
Between Groups	0.29	2	0.14	3.92	0.02
Within Groups	2.40	66	0.04		
Total	2.69				

In Figure 9, the average density, percent anagen and growth rates are depicted for each test material, showing the overall efficacy of each product in promoting hair growth.

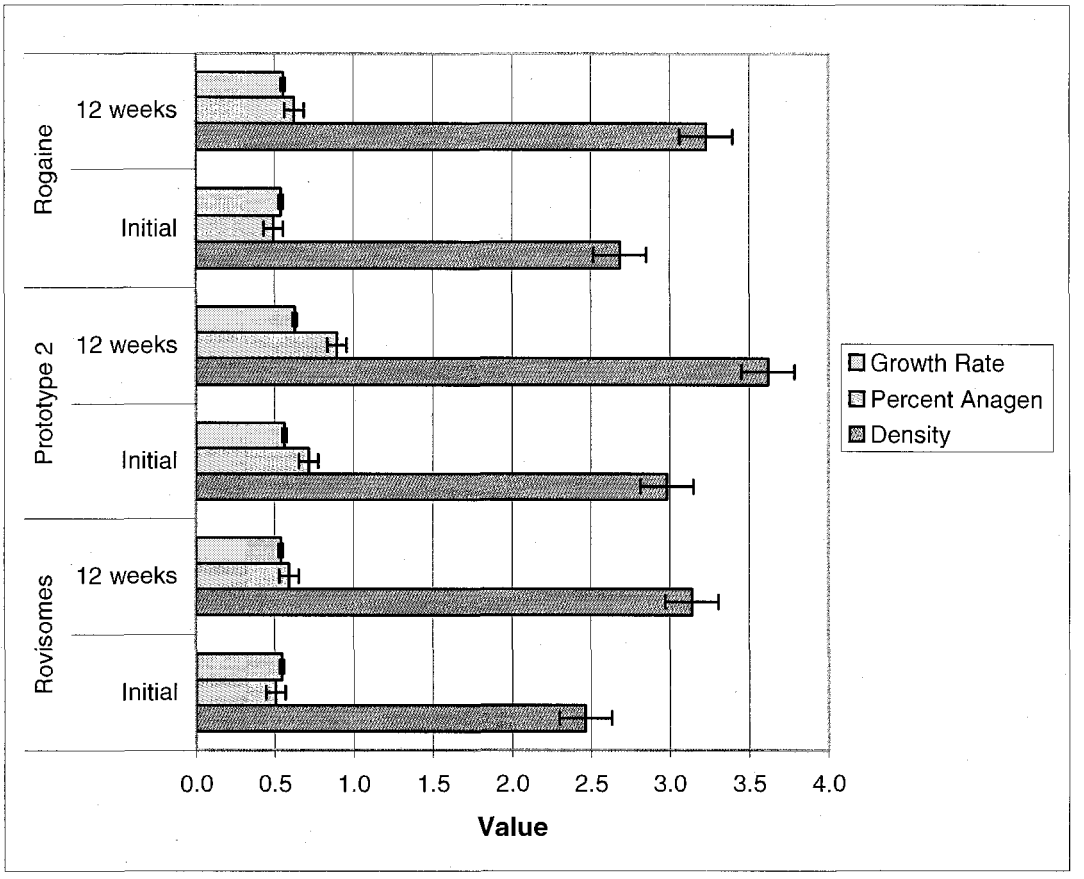


Figure 9- Comparison of Average Values (+/- SE) for Objective Measurements for all 3 test products

From Figure 9, it is evident overall density increased from baseline to study conclusion for all three-test products. Also, the number of hair follicles in the anagen stage increased for all test variables, but for growth rate, Rovisomes was stagnant, while Prototype 2 and Rogaine® showed slight increases.

As a means of looking for trends within the cells of the three test products, and to verify adequate stratification of cell demographics, correlation tables were created for identifying potential relationships. These are shown in Tables 23 – 25, and possible strong associations ($r > 0.5$) are depicted in bold.

Table 23- Correlations within Rovisomes Cell (All r values greater than +/- 0.5 bolded)

Category	Age Decade	NH Score	Years Bald	Ini. % Ana	12wk % Ana	% Change Ana	Ini Dens	12wk Dens
Age Decade	1							
NH Score	0.253	1						
Years Bald	0.490	0.380	1					
Ini. % Anagen	0.056	0.377	0.163	1				
12wk % Anagen	0.228	-0.027	0.065	**0.555**	1			
% Change Anagen	-0.031	-0.481	-0.150	**-0.750**	-0.080	1		
Ini Density	-0.413	-0.286	-0.285	0.093	0.331	-0.013	1	
12wk Density	-0.189	**-0.514**	-0.498	0.039	**0.584**	0.313	0.490	1
% Change Density	0.117	-0.289	-0.325	-0.074	0.305	0.409	-0.328	**0.637**

Table 24- Correlations within Prototype 2 Cell (All r values greater than +/- 0.5 bolded)

Category	Age Decade	NH Score	Years Bald	Ini. % Ana	12wk % Ana	% Change Ana	Ini Dens	12wk Dens
Age Decade	1							
NH Score	0.002	1						
Years Bald	0.244	0.474	1					
Ini. % Anagen	-0.435	-0.486	-0.361	1				
12wk % Anagen	-0.147	-0.438	-0.474	**0.746**	1			
% Change Anagen	**0.576**	0.227	0.080	**-0.727**	-0.140	1		
Ini Density	-0.358	-0.443	-0.287	**0.834**	**0.701**	-0.498	1	
12wk Density	-0.223	-0.396	-0.321	**0.715**	**0.762**	-0.268	**0.889**	1
% Change Density	0.381	-0.080	-0.058	-0.194	0.169	0.449	-0.162	0.287

Table 25- Correlations within Rogaine® Cell (All r values greater than +/- 0.5 bolded)

Category	*Age Decade*	*NH Score*	*Years Bald*	*Ini. % Ana*	*12wk % Ana*	*% Change Ana*	*Ini Dens*	*12wk Dens*
Age Decade	1							
NH Score	0.009	1						
Years Bald	0.194	**0.625**	1					
Ini. % Anagen	-0.182	**-0.501**	-0.341	1				
12wk % Anagen	-0.053	-0.452	-0.301	**0.777**	1			
% Change Anagen	0.138	-0.021	0.049	-0.060	**0.563**	1		
Ini Density	-0.247	**-0.536**	-0.294	**0.805**	**0.674**	0.034	1	
12wk Density	-0.318	**-0.525**	-0.415	**0.793**	**0.742**	0.163	**0.914**	1
% Change Density	-0.153	-0.092	-0.217	-0.034	0.152	0.317	-0.186	0.199

Each cell exhibited both positive and negative strong correlations, particularly in the Hamilton-Norwood score relative to percent anagen and density values. The initial and twelve week percent anagen categories also had strong relationships with density values and percent anagen changes. The age decade, years balding and the percent change in anagen had minimal relatedness for each of the three test products. Scatter plots (data not shown) of all interacting categories verified the absence of influential points in creating potential false correlations.

The results from the self-perceived assessments evaluated at the conclusion of the study are compared in Table 26.

Table 26- Average Self-Perceived Assessment of All Hair Growth Test Products

Attribute / Product	Rovisomes	Prototype 2	Rogaine®	ANOVA *p*-value
Overall Liking	6.57	5.74	6.50	0.13
Aroma	6.00	5.26	5.56	0.30
Easy Dispensing	7.48	7.68	7.76	0.66
Quick Absorption	7.10	7.26	6.59	0.26
Enhanced Natural Hair	5.05	4.58	5.65	0.24
Improved Hair Appearance	5.19	4.74	5.53	0.37
Improved Texture	5.14	4.53	5.06	0.50
Improved Density	5.38	4.37	5.28	0.20
Concept was Intriguing	6.81	6.29	7.25	0.32

For the each of the above attributes, there was no significant difference in rating value across the three product types. For Prototype 2, overall liking and aroma of the product was rated below Rovisomes and Rogaine®, but was at near parity with Rogaine® for dispensing onto the scalp and directionally better than the other two variables at absorbing into the skin following application. In terms of improving hair growth and the physical characteristics of the hair (appearance, texture and density), Prototype 2 was consistently rated lower than both Rovisomes and Rogaine®. This was also the case for the perceived intrigue regarding the product concept.

CHAPTER VI: DISCUSSION

The screening of extracts across four bioassays resulted in the creation of a testable and usable hair growth prototype formulation to potentially treat androgenic alopecia. The novelty of screening botanical samples for efficacy in VEGF, KGF, proteasome and IL-1α bioassays to develop an end product was enhanced by the use of DOE software. Incorporation of the approved extracts into a liposome formulation proved to be successful, since particle size and aesthetic attributes were acceptable even after a long-term stability test.

However, flaws were also exposed in cell culturing techniques and use of an arbitrary scoring system to successively screen botanical extracts in a rapid fashion. Since much of the human HF biology work is done on cells obtained from elective, cosmetic surgical procedures (Lu et al., 2006; Xiong and Harmon, 1997; Philpott et al., 1990), it is necessary to have full accounts of the age, gender information of the donors, as well as isolation locations of commercially available HF cells. During the course of the *in-vitro* screening process, variability was often observed in the responses elicited by the botanical samples. As the screening of extracts for this project occurred over a one-year span, the cell culturing practices were evolving due to an increasing staff and corporate emphasis. Within that time, it was discovered much of the initial HFDPC culture work was conducted on either female-derived tissue, and/or had been undergoing varying periods of frozen storage states prior to flask and plate seeding as well as different cell-passage techniques. Such conditions may have influenced how the screened extracts performed on each assay. In addition, if HFDPC cells have been allowed to progress into later passages of culture (6+), they undergo transformation and

have little resemblance to *in-vivo* counterparts (Randall, 1996). Therefore, if all extracts had been screened consistently, in terms of HFDPC type and passage, it is totally plausible to have arrived at a completely different blend of extracts and to have eliminated some the variability observed in cytokine and growth factor expression. Furthermore, expression levels of the growth factors were significantly reduced from initial runs (data not shown). Ideally, all extracts should have been tested the same number of times to fairly assess variability and performance. Unfortunately, time and cost were negative factors prohibiting this from occurring. The cell-free proteasome assay was not affected by any of this variability.

The addition of the IL-1α assay to the extract screening process may have also acutely focused overall scoring results, but this assay was included just prior to the DOE analyses. Before the use of IL-1α, a dihydrotestosterone assay was utilized as an extract-screening tool. This androgen has pertinent regulatory control over hair growth and can eliminate scalp hair fiber production (Mulinari-Brenner and Bergfield, 2001). This assay was stopped due to inconsistencies with the ELISA kit (data not shown) and the discovered ineffectiveness of topical applications inhibiting dihydrotestosterone activity.[3] IL-1α is still somewhat controversial in terms of hair loss causation and cures, with a large body suggesting it inhibits hair growth (Stenn and Paus, 2001) and causes atrophy of the HF in *in-vitro* culture experiments (Mahe et al., 1996). Recent work, however, indicates it may up regulate VEGF and KGF, which are favorable to hair growth (Boivin et al., 2006). Despite its controversial status, IL-1α still remains a critical cytokine in terms of treatment, since it is a good marker for inflammation, a condition not to be

[3] Outside consultation led to this discovery.

overly promoted during topical treatment. When using this marker in the DOE, the focus was on selecting extracts at concentrations that did not up regulate IL-1α in a substantial way, but rather, were comparable to media treated (control) cells.

For all DOE experiments, cell source and passage were known and documented to coincide with actual male-pattern baldness, undergoing two cryofrozen states maximally and treated at passages four and five only. In DOE 1, the contradictions in extract performance compared to the initial screening process were evident for VEGF (Extracts Lichochalcone, Green Rooibos and Saw Palmetto) and KGF (Lichochalcone), while proteasome performance matched nicely. Since VEGF and KGF are cell-based assays, and the proteasome assay is strictly enzyme-based, it is possible the combination of the living systems and the aforementioned discrepancies in technique contributed to the variation observed between screening and DOE analysis. Similar trends were seen in DOE 2, thereby indicating the DOE experiments have greater validity at elucidating extract activity in the assays than the initial screening process.

In regards to the extracts selected for incorporation into Prototype 2, all four have noted properties slated to be beneficial to humans. Additionally, this select blend of extracts incorporated into a topical treatment for hair loss, specifically targeting the modulation of VEGF, KGF, IL-1α and the proteasome, is unique and novel.

Rooibos, a native South African legume (*Aspalathus linearis*), is commonly used as a tea and has been found to contain relatively high concentrations of polyphenolic antioxidants. Such compounds can inhibit free-radical damage, common in coronary diseases and cancers. Folklore traditions have claimed Rooibos as curing colic and soothing various skin allergies (Erickson, 2003). Additional benefits include microbial

resistance, anti-aging and inflammation mediators in the oral cavity and within muscle and joint tissue (Cosmetics Design, 2006).

Lichochalcone, specifically Lichochalcone A, is licorice root extract from *Glycyrrhiza inflata.* It has long been used as a traditional medicine in eastern cultures, treating digestive ailments and allergic conditions (Shibata, 2000). Benefits of applying Lichochalcone topically include reduced irritation from both mechanical and ultra-violet stimuli (Kolbe, et al., 2006) as well as acting as an anti-inflammatory treatment (Cui, et al., 2008). Lichochalcone A, as supplied for this research, has been noted as also having anti-microbial properties and inhibiting the hair growth related enzyme, 5-α reductase, as well as androgen activity (Barnet Products Corporation, 2007).

Shiso extract is isolated from *Perilla ocymoides* leaves and has been generally used for both medicinal and dietary purposes in the Far East. Claims include anti-bacterial efficacy, treatments for digestive disturbances and diseases of the skin, with clinical data indicating improvement of dermatological conditions (Barnet Products Corporation, 2005).

Saw Palmetto lipid extract is isolated from the berries of *Sabal serrulata,* a native small palm of the southern United States. It is a Native American traditional medicine and is a common botanical treatment for benign prostate hyperplasia. Inherent phytosterols mimic the androgens testosterone and dihydrotestosterone and infer anti-androgenic properties to the extract (Euromed, 2000).

Formation of liposomes containing maximally allowed levels of these extracts was not possible due to incompatibility and inverse solubility characteristics between Lichochalcone, Green Rooibos, and possibly Saw Palmetto. In addition, the hydrophilic

and hydrophobic properties of the extracts, at the highest allowed concentrations, impeded liposome formation, resulting in higher concentrations of lecithin usage to mediate hydrophobic extract incorporation. Despite the limiting extract concentration factor obtained from the Repeat Insult Patch Testing, all four selected extracts were incorporated into Prototype 2 by utilizing all three phases (oil, water and external) during development. Three passes through the microfluidizer resulted in optimal liposome particle size, which remained relatively constant throughout elevated temperature stability testing. This successfully demonstrates stable liposomes and stable external phases in the prototype formulation.

The pH profile was out of the specification range, but that occurred during the production process most likely due to insufficient addition of Arginine, which was being utilized as a pH booster. An additional twenty-five to fifty percent increase in Arginine is expected to bring the final product pH into the ideal slightly acidic (Jung et al., 2006) specification range. Since the overall pH range varied only slightly throughout the stability test, it is reasonable to expect that a formulation within the proper pH range would also remain stable over time and temperature variations.

The discolorations of the product at higher temperatures, as well as the fragrance character, are not atypical results for this product type. Color often degrades at higher temperatures, but the changes observed in Prototype 2, at both 50°C and 40°C, still allowed the formulation to retain a brownish character, with no evident precipitation. Likewise, the fragrance character changed slightly at the elevated temperatures, most likely due to slight rancidity of lipid components in the formula. By incorporating an actual fragrance into the prototype, this could easily be masked.

Through the novelty of screening botanical extracts in four bioassays, and the optimization analyses by the DOEs, a potential hair growth promoting formulation has been developed. In the process, modification of cell-culturing techniques and understanding of the variability in living systems has led to additional insight on hair follicle dynamics. Future botanical extracts, in addition to Lichochalcone, Shiso, Saw Palmetto and Green Rooibos, may be more efficiently screened for efficacy on *in-vitro* hair follicle systems, thereby leading to additional testable *in-vivo* products targeting hair growth restoration.

Minoxidil, the active ingredient in Rogaine® Extra Strength, has been known to be a promoter of scalp hair growth, as a result of topical application, since the early 1980's (Vanderveen, et al., 1984). Since it is only one of two Food and Drug Administration (FDA) approved drugs to treat hair loss (Leavitt, et., al., 2005), it has become the benchmark for topically applied products designed to combat androgenetic alopecia. Finasteride, the other approved compound, is orally administered (Merck & Co, www.propecia.com., 2007) and its efficacy is irrelevant, as such, to the scope of this research.

Early examination of minoxidil's effectiveness at treating hair growth in androgenetic alopecia shows limited success. Only three of five minoxidil users experienced notable hair growth using a 5% concentration, with only one rated as having "appreciable restoration of larger, thicker pigmented terminal hair" (Vanderveen et al., 1984, p. 418) and the other two noticing a slight increase in terminal hairs. The other two individuals had no hair regrowth, but these were 1% minoxidil users. These evaluations

included both subjective evaluations and scalp biopsy analysis with emphasis on hair shaft diameter (Vanderveen et al., 1984).

More recent examinations of minoxidil's performance included trials comparing varying concentrations of minoxidil (Olsen et al., 2002) and minoxidil delivered in different forms (Shin et al., 2007 and Olsen et al., 2007). A 5% versus 2% minoxidil concentration study showed the higher active test product to be more efficacious than the lower concentrated material. After forty-eight weeks of usage in men aged 18-49, the 5% material resulted in 45% greater non-vellus hair regrowth in the AOI compared to 2% minoxidil results. Additional parameters, assessed by both the patients and expert evaluators, all indicated the 5% minoxidil was better at increasing scalp hair coverage, and having positive impacts on self-perception due to increased hair growth/regrowth. The AOI hair counts were done by macro photography with manual conversion of terminal hairs to acetate overlays, followed by image software counting (Olsen et al., 2002)

When 5% minoxidil was incorporated into a hydroalcoholic foam base and tested versus a placebo, a significant increase in hair counts occurred in the minoxidil version. This 16-week study included 352, 18-49 year old males, with Hamilton-Norwood balding scores ranging from III-V, and with an average duration of balding being fourteen years. Target area hair counts in the AOI (using similar measurement techniques as the previous study above) increased by approximately 345%, over the placebo, by the end of the sixteen-week study. Subjective self-assessments were also significantly better for those using the active product, with 70.6% of minoxidil users claiming an improved condition to the placebo's 42.4%. In neither case, the age, balding score, nor years balding had any

influence on the results. Lastly, global scalp photographs, when rated by expert graders, also showed significant increases in hair growth for minoxidil users versus the control patients (Olsen et al., 2007).

A study employing similar evaluation techniques as used in this research was conducted with 5% minoxidil combined with the skin penetration enhancer tretinoin. A topical solution containing just 5% minoxidil served as the control. A total of thirty-one males between 28-45 years of age, with Hamilton-Norwood balding scores ranging from III-V, participated. No significant difference existed between the two treatments in total hair counts, anagen hair ratios, growth rate and self-assessments (Shin, et al., 2007).

A joint clinical-like study between Synymed Incorporated and the University of California San Francisco evaluated a non-liposomal cream-based herbal topical treatment on androgenetic alopecia males. This herbal blend included fennel, polygonum, mentha, chamomile, thuja and hibiscus. Twenty-four participants were using either the 7.5% herbal cream or a placebo cream. All subjects were under 55 years of age, and had balding scores between III-IV. The parameters evaluated were total and terminal hair counts, hair length and total hair weight. Average percent changes were significant for total and terminal hair counts (77.4% for active vs. 3% placebo and 169.4% for active vs. 33.9% placebo, respectively), while those for total hair weight and average hair length were not statistically different between the test material and the placebo (Greenberg and Katz, 1996).

The Rovisomes material tested in this research project contains the active ingredients of biotin (Vitamin H), linoleic acid (Vitamin F), Vitamin E-acetate, D-panthenol (Provitamin B5) and caffeine all incorporated into a liposome carrier system to

enhance skin penetration. Results of a limited twenty-four week study showed increases in hair density and the number of hair follicles in the anagen stage of the hair cycle. For the six males participating, a 9.4% increase in density was calculated, while anagen hair follicles increased by 5.3% and telogen follicles decreased by 16%. Self-assessment scores, converted to a 9-point scale used within this body of research, were as follows: formulation consumption- 7.0; formulation spreading- 7.4; hair quality- 7.2; hair volume- 6.9; and overall rating- 7.2. Statistical comparisons are not mentioned and separation of the self-assessment scores between male and female users is not given (Rovi Cosmetics, "An innovative serum for increased hair density" is an internally submitted report).

The value of the research conducted herein can only be appreciated if placed in the context of previous attempts to promote hair growth in those suffering from androgenetic alopecia, as referenced above. The herbal blend of Lichochalcone, Shiso, Green Rooibos and Saw Palmetto incorporated into a liposomal vehicle has shown comparable success to the leading topical treatment, Rogaine® Extra Strength (5% minoxidil). Hair density, number of anagen (active) follicles and growth rates increased from study commencement to its conclusion for all three test products, and these increases were all statistically significant. While the Rovisomes material was directionally better at increasing hair density, the Prototype 2 formulation yielded directionally better results than Rogaine® and Rovisomes for increasing anagen hair follicles and improving growth rates, with the change in growth rate being statistically different versus Rovisomes. It is reasonable to conclude all three-test products, to some degree, had hair growth promoting effects as evidenced by the objective measurements.

As this research included expanded age ranges (18-59), the whole spectrum of Hamilton-Norwood balding scores (1-8) and longer durations of experiencing a balding condition (1-35), the elicitation of positive objective responses is excessively burdened. However, the Prototype 2 material increased hair density by 24%, promoted anagen hair follicle presence by 36.4% and boosted hair growth rates by 12.2%. These changes, though not as impressive as some of the figures cited above, are respectable given this work's broader demographics and differences in measurement techniques. This is further supported by Rogaine's® performance- a 25.4% increase in density, 27% increase in anagen follicles and only a 3.1% increase in growth rate. Even though the Rovisomes material performed directionally better with a 32.4% increase in density and 30.1% increase in anagen hair follicles, its negative growth rate of –3.3% and absent discussion pertaining to measurement methodology and limited male participation question the relevant efficacy of this product.

Subjectively, even though not significantly different, the Prototype 2 formulation trended downward with the exception of product dispensation and absorption of material into the scalp, where it was rated as second best and first, respectively. Even though true objective measurements can remove personal bias, for hair growth promoting treatments, the subjective perception is the critical criteria for ultimately determining product success (Piérard et al., 2004). In this regard, Prototype 2 was deficient, particularly in terms of positively influencing hair quality. However, a recent editorial by Dominique Van Neste, contrasts objectivity to subjectivity in hair growth evaluation, with detection of a worsening balding condition taking longer to recognize using subjective evaluations than objective assessments. Furthermore, the self-perceived evaluation is deemed "the least

effective" assessment tool while a contradictory notation between objective and subjective values in a study comparing the two benchmark products, Rogaine® and Finasteride, highlights this discrepancy (Van Neste, 2008). Participants must be given the tools to self-evaluate, as well as an appropriate amount of time. In this study, individuals were not officially given before and after global images of their scalp to decide on their product's efficacy. Self-evaluation in a mirror and commentary from casual observers were the tools used to rate product performance. Neither tool may be extremely accurate, and the latter may artificially inflate performance should it be suggestive of an increase in hair growth (Van Neste, 2008). Furthermore, the protocol of cutting the entire scalp hair to ¼" length, and maintaining this length throughout the study, does not lend itself to a comprehensive self-evaluation since changes in hair density, active follicles and growth rate cannot be accentuated at the normal hair length. All three-test products were subject to these potential impedances of self-assessment, and as a result, the above ratings could potentially be skewed.

From the correlation tables, there appears to be no major revelations regarding demographic stratification or product influence on the balding condition. For instance, in the Rovisome's group, a strong negative correlation (-0.514) exists between balding score and hair density. Since higher the balding score, the less hair present, it is reasonable to conclude density will also be minimal in advanced balding states. A similar, though positive, pattern (0.625) exists in the Rogaine® cell for the number of years balding and Hamilton-Norwood score. Inspection of relationships between the percentage of follicles in anagen over time and density changes are reasonable. Having a relatively large number of follicles in anagen at the conclusion of the study coincides with having a

larger percentage of follicles in anagen at the study onset. This was the case for all products and these were all potentially strong positive correlations (0.555 for Rovisomes, 0.746 for Prototype 2 and 0.777 for Rogaine®). Given the length of time required to notice changes in hair loss (Van Neste, 2008) it is also not surprising to have negative correlations with initial anagen follicles and the change in the number of anagen follicles over a three-month period. These were strong for both Rovisomes (-0.750) and Prototype 2 (-0.727). Finally, if the number of follicles in anagen is high, then density should also be greater, as was the case for Rovisomes (0.584), Prototype 2 (0.701 and 0.762) and Rogaine® (0.674 and 0.742).

The in-house clinical portion of this research utilized equipment and computer software already available to the research team. Even though convenient, the on-hand technology did not allow for the evaluation and measurement of all non-invasive parameters deemed essential for tracking hair growth response to topical treatment. Generally, hair growth analysis methods should be able to capture hair density (fibers/square area), hair fiber diameter, hair growth rate and the anagen-telogen ratio (Hoffmann, 2001). Hair fiber diameters were not measured due to variability in image quality, limits on the magnification of the AOI's and software measuring capabilities. Hair density was captured and measured as pixel concentration within the AOI. Anagen and telogen ratios, as well as growth rates, were manually tagged on digital images and were again influenced by image quality. More automated techniques are available, notably the TrichoScan system, which combines epiluminescent microscopy and complete digital analysis to track all essential changes in hair growth (Hoffmann, 2001 and Hoffmann, 2005). However, due to cost issues and availability, such noted methods

and technology could not be employed in this study. Furthermore, the adaptations described here were applied to all product cells and analysis photos, so bias of methodology was limited. It is also worthwhile to note systems such as TrichoScan, even though popular for clinical analysis, still have noted detractors (Van Neste and Trueb, 2006), with implication that no tool for hair growth analysis is flawless.

Additional factors involved in this protocol include modifications to the traditional phototrichogram and contrast enhanced phototrichogram methods. In both of these procedures, the AOI is minutely, but permanently tattooed so as to be able to definitively relocate it throughout the study duration. The hair within the AOI is also temporarily dyed up to twelve minutes so as to artificially pigment unpigmented hair such as vellus or gray hairs. Vellus hairs can then be differentiated from terminal hairs by measuring hair diameters (Hoffmann, 2001 and Van Neste, 2001). The protocol employed in this research was restricted to non-invasive techniques and involved capturing coordinate measurements from the bridge of the nose to the point of ear attachment and marks made with "permanent" ink. Marked locations would rarely remain for the three-day follow-up measurements, let alone the full twelve weeks. Hair dyeing was performed but left on for only two minutes, since any longer duration resulted in excessive scalp staining, even after wash out. The darkening of the skin within the AOI impeded accurate density measurements, and counting of anagen-telogen follicles. Any future analysis should consider permanent tattooing to hallmark AOIs and more uniform dyeing techniques to ensure complete hair color transformation without scalp penetration.

The clinical portion of this study incorporated the botanically derived and liposomal-based Prototype 2 formulation, with comparisons made to the liposomal Rovisomes and the topical gold-standard treatment, Rogaine®. All three products exhibited success at improving hair density, increasing the number of anagen follicles or increasing the rate at which scalp hair grew. From an objective analysis viewpoint, all three-test products produced significant improvement in hair density and the number of anagen follicles, compared to baseline values. Prototype 2 and Rogaine® both increased growth rate from baseline measurements, but with no statistical significance. Subjectively, Prototype 2 was the lowest rated product for improving hair quality. Since this was the first attempt to validate the efficacy of this patent-pending technology *in-vivo* any future work should aim to improve its aesthetic properties. Likewise, the *in-vitro* claim that this specific blend of Lichochalcone, Shiso Extract, Saw Palmetto and Green Rooibos modulates VEGF, KGF, IL-1α and the Proteasome, as they relate to hair growth, should be verified for *in-vivo* proof of concept. Should that claim prove to be successful, coupling future clinical assessments with improved techniques and technology, could potentially brand Prototype 2 as a topical treatment to treat androgenetic alopecia.

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