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Human Hair Histogenesis for the Mitochondrial DNA Forensic Scientist

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ABSTRACT: Analysis of mitochondrial DNA (mtDNA) sequence from human hairs has proven to be a valuable complement to traditional hair comparison microscopy in forensic cases when nuclear DNA typing is not possible. However, while much is known about the specialties of hair biology and mtDNA sequence analysis, there has been little correlation of individual information. Hair microscopy and hair embryogenesis are subjects that are sometimes unfamiliar to the forensic DNA scientist. The continual growth and replacement of human hairs involves complex cellular transformation and regeneration events. In turn, the analysis of mtDNA sequence data can involve complex questions of interpretation (e.g., heteroplasmy and the sequence variation it may cause within an individual, or between related individuals). In this paper we review the details of hair developmental histology, including the migration of mitochondria in the growing hair, and the related interpretation issues regarding the analysis of mtDNA data in hair. Macroscopic and microscopic hair specimen classifications are provided as a possible guide to help forensic scientists better associate mtDNA sequence heteroplasmy data with the physical characteristics of a hair. These same hair specimen classifications may also be useful when evaluating the relative success in sequencing different types and/or forms of human hairs. The ultimate goal of this review is to bring the hair microscopist and forensic DNA scientist closer together, as the use of mtDNA sequence analysis continues to expand.

KEYWORDS: forensic science, hair biology, hair development, forensic hair comparison, hair microscopy, hair DNA, forensic DNA, mitochondrial DNA, mtDNA, mtDNA heteroplasmy

The analysis of mitochondrial DNA (mtDNA) has been used in hundreds of forensic cases over the past ten years, including some high profile historical cases (1). While the usual spectrum of biological specimens are candidates for mtDNA analysis, human hairs are most often the evidentiary source in criminal investigations. However, only those hairs that are deemed unsuitable for nuclear DNA (nucDNA) typing are considered, as nucDNA analysis provides greater discriminatory potential (2). In general, hairs that do not have a growing root, or adhering tissue on the resting root, are unsuitable candidates for nucDNA analysis (3). However, questions of suitability may arise since it is unclear exactly how much root material is required for nucDNA analysis. Individual hair root sizes will vary considerably, and fluctuations in the amount of hair root follicle remnants are common. Thus, in its simplest form, suitability is established by determining whether the hair root contains soft tissue. Hair follicle sheaths, follicular tags, and growing root stems are all sources of soft tissue. In contrast, the resting hair root is composed of keratin, a poor source of nucDNA. Nevertheless, follicular sheaths or tags that are suitable for nucDNA analysis can accompany resting roots, and identification of these microscopic structures is possible with minimal training. Absent microscopic evaluation, suitability can also be determined empirically by attempting nucDNA analysis. While this is a destructive process, and will consume a portion of the DNA extract, if nucDNA typing can be performed it will enhance the strength of the DNA evidence. Before such analysis, the forensic scientist should consider quantifying the amount of DNA recovered (4), as this will be an initial indicator of success for nucDNA analysis.

Forensic mtDNA analysts are primarily interested in the DNA content of the hair shaft rather than the hair root. Nuclear cellular material (with the accompanying nucDNA) is apparently degraded during the keratinization process of the human hair shaft. In contrast, mitochondria are commonly observed components of the keratinizing hair shaft (5). Since there are multiple copies of mtDNA in each mitochondrion (hundreds of mitochondria and thousands of copies of mtDNA in each cell, compared to two copies of nucDNA) (6,7), the presence of mitochondria in the hair shaft serve as a rich source of mtDNA. In addition, this high-copy-number characteristic of the mtDNA genome means that other biological sources that do contain nucDNA, but are highly degraded or environmentally challenged, are suitable candidates for mtDNA analysis.

Microscopic Evaluation of Hairs

Prior to DNA analysis, it is recommended that hair and pubic hairs be subjected to microscopic identification and comparison to a known sample. All hair DNA extraction techniques render that part of the hair unsuitable for subsequent microscopic comparison. The questions of possible forced removal of the hair, indicating struggle, and possible cosmetic treatments may arise after DNA extraction involving the root portion. The majority of hairs recovered from items submitted to public forensic laboratories do not ultimately associate a suspect to a victim, or a suspect to a crime scene. Most of the recovered hairs are determined by comparison microscopy to have origin from the victim. In a study conducted by one of these authors (CAL), sexual assault victim pubic hair combings were found to contain hairs microscopically dissimilar (foreign) to the victim's known pubic hair sample in only about 2% of the cases (unpublished data). Contemporary hair examiners require at least 25 pulled exemplar hairs (head and/or pubic) from a known

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individual for comparison to a single evidence hair since the natural range of microscopic variation found in one individual's hair can be substantial (e.g., a person who appears to have red head hair, may have predominate brown, blonde, and gray hairs, with a minor population of red hairs in their scalp) (8). Hair examiners have recognized that in some cases the microscopic characteristics in an individual's hair sample may have changed with time and could possibly lead to a false exclusion of a victim or suspect (9). Labial hairs, scrotal hairs, and chest hairs can have very different microscopic characteristics than the pubic hairs from the same individual, but can appear to have pubic origin when viewed individually. When these hairs are dislodged from the victim or the suspect and recovered in the victim's pubic hair combings, they are also candidates for false exclusion by microscopy because they are assumed to be pubic hairs, and are only compared to known pubic hairs. Furthermore, limb, trunk, and facial hairs are not usually compared microscopically since they can have too few distinctive microscopic characteristics, but may have potential value as DNA specimens. Thus, the ability to associate a hair with an individual microscopically should not be the sole prerequisite for DNA analysis. Instead, the microscopist should be conscious of possible false exclusion factors when performing microscopic comparison to known and questioned hairs prior to rejecting any possible suggestion of subsequent DNA analysis. Nonetheless, comparison transmitted light microscopy remains the most timely and cost effective method to determine which hairs in a case should be referred for possible DNA typing.

In any case, a microscopic biological profile of the evidence hair should be obtained prior to analysis, whenever possible. Some animal hairs, natural fibers, and synthetic fibers can be macroscopically identical to human hairs. The biological profile should include species identification, probable somatic origin, probable racial origin, and root morphological classification. This information will assist in determining whether nucDNA or mtDNA analysis is possible, and whether the results may be probative (e.g., a recovered Caucasian facial hair would not be referred for DNA analysis if the suspect was known to be Asian, Black, or Caucasian with no facial hair.)

Mitochondrial DNA Heteroplasmy

The major source of complexity that has arisen from the analysis of mtDNA data from hair shafts involves sequence heteroplasmy (1). Heteroplasmy is defined as a heterogeneous pool of mtDNA molecules in the cytoplasm of the cell; hence the derivation of the word *hetero-plasmy.* In more general terms, heteroplasmy is a mixture of multiple (usually two major) mtDNA sequence types in a single individual. The heteroplasmy, while not established empirically, is most likely distributed randomly between different mitochondria, and this random distribution most likely extends to the cellular level. This has been substantiated by the fact that low level control region (CR) heteroplasmy has been observed at a relatively high rate in tissue sources separated by developmental differentiation (10). Thus, if high quantities of low level sequence heteroplasmy exist, the mtDNA sequence of one isolated population of cells may be different than the sequence of a second population. As a result, heteroplasmy interpretation can be a complex process that requires knowledge of the biological forces that are driving the distribution of mtDNA in the cell, and the relation of these factors to the cellular characteristics of the hair.

The inheritance pattern of mtDNA is strictly maternal (11), despite some recent suggestions otherwise (12,13). As a result, it had generally been thought that an individual inherited a single mtDNA haplotype from their mother, and that observed heteroplasmy in the coding region of the genome was due to somatic deletions throughout an individual's lifetime. In fact, the first reported observation of mtDNA heteroplasmy in humans was cited in 1988 (14), and involved large deletions in the coding region. However, the first reported observation of heteroplasmy in the CR did not occur until 1994, and did so in a forensic setting (15). The heteroplasmy reported in that forensic study was at a single nucleotide position, and did not involve a deletion event. Since then, multiple observations of CR heteroplasmy (both nucleotide and length-based) have been cited.

While the complexities of interpreting heteroplasmy data from hairs in a forensic setting have been documented (16), knowledge of human hair histogenesis provides a foundation from which to evaluate the typical and atypical case results. Consideration of the unique events that occur during hair development and regeneration are useful not only for the forensic microscopist, but also for the forensic mtDNA scientist. This knowledge can be used as an aid in avoiding false exclusions (as in the latter case, triggered by sequence heteroplasmy and/or apparent substitution events). The potential for subtle variations within similar sets of mtDNA sequence data can be better understood and described if one has a thorough knowledge of hair histogenesis. However, such evaluations require a consideration of the human hair at the cellular level. There have been no reviews in the literature that specificaly trace the continuous migration of mitochondria in cells from the new hair bulb to their ultimate destination in the keratinized hair shaft. Therefore, this article attempts to describe in detail the origin and path of the mitochondria that are contained in the human hair shaft, and attempt to relate these biological characteristics to the interpretation of mtDNA data, including sequence heteroplasmy.

Hair Embryology

Hair is often referred to as a thread of protein growing from follicles in the skin of mammals. More specifically, hair can be described as an epithelial product of a multicellular primitive epithelium. The primitive epidermis of a human embryo begins to organize clusters of cells for the production of hairs at about nine to twelve weeks gestation (Fig. 1). A discrete group of cells gather in the primitive basal zone (ectoderm) of the skin and begin a downward growth pattern to form a hair peg. Keep in mind, the term hair peg can be misleading because it is actually the precursor of only a portion of the follicular apparatus, as there is a second gathering of cells below the hair peg in the primitive mesoderm. The epithelial hair peg grows downward to meet this ball-shaped cluster of mesoderm cells that will ultimately form the dermal papilla as well as the fibrous sheath of the follicle. Through growth factor secretion, the dermal papilla influences the hair forming matrix cells in the hair bulb, but no cells are contributed to the hair shaft from the dermal papilla. Nonetheless, the onset of primary folliculogenesis is random, producing a mosaic of follicles in different stages of development in the fetal skin. These follicles retain their independent cycling patterns into adulthood where each hair follicle's growth phase is independent of its neighbors.

Once established, the fetal epithelial hair peg enlarges into a bulbous mass of cells that enclose the primitive dermal papilla. This arrangement ultimately forms the follicle that will produce the hair. In addition, these ectodermal (hair bulb) and mesodermal (dermal papilla) cellular elements remain in intimate contact throughout the lifetime of the individual during stages of rapid hair growth, re-

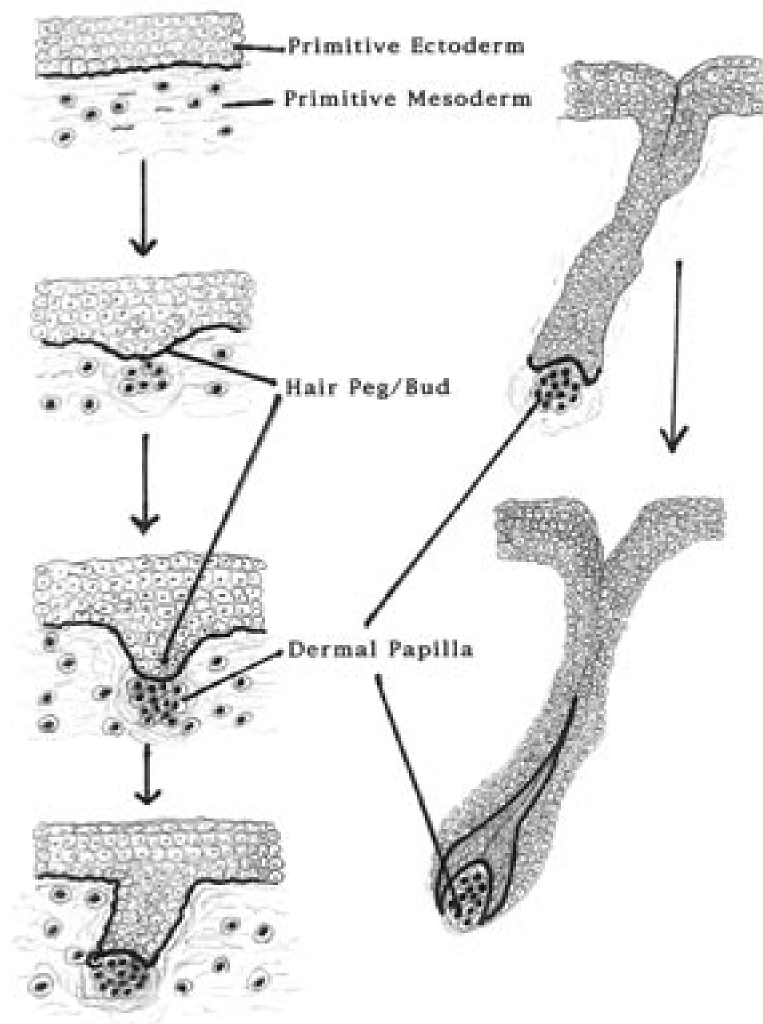

FIG. 1—*Embryonic hair development. Left, progressive differentiation of cells in fetal skin to form the hair peg. Right, final formation of hair follicle from which the first hair will grow. This follicle will continuously regenerate itself producing new hairs throughout the lifetime of the individual.*

gression, rest, and regeneration. By 16 to 20 weeks gestation, the embryonic hair follicles may be producing hairs (5). The hair follicles established at this point will be all that the individual will have during their lifetime. Thus, the cells that feed the growing hair shaft with mitochondrion are clonal in nature, and the pool of mtDNA molecules will reflect this isolated population.

Following embryogenesis, the average person is estimated to have approximately 5 million body hair follicles (5). Although the follicle number remains constant, the size of individual follicles, and any resulting hairs, can change dramatically with time. This is primarily due to the influence of androgens. For example, fine hairs on the pubis change to coarse terminal pubic hairs during puberty and coarse head hairs revert to fine hairs on the scalp in androgenetic alopecia (17).

Hair Histology

The hair dermal papilla is composed of primary fibrocytes of mesodermal origin, and it directs the mitotic activity (i.e., cell division) of the adjacent ectodermal matrix cells that constitute the hair root bulb (Fig. 2). Although any follicular material adhering to a plucked hair bulb, or hair root club, is usually of ectodermal origin, in cases where a complete bulb is present, one needs to consider the additional possible existence of mesodermal tissue from the dermal papilla. Most importantly, hair root bulbs and follicular tissue would contain nucleated cells providing a source of nucDNA for analysis.

The hair shaft fiber is of ectodermal origin, as it is fed through the cellular activities of the hair bulb matrix. The hair bulb matrix has been described as a boiling kettle with the bubbles representing the cells that form the hair shaft. (This analogy may be useful for expert witnesses to describe the hair growth process to juries.) Cells in the root bulb divide once every 23 to 72 h (5), and are arranged in a concentric fashion, immediately above the dermal papilla. Thus, one could view the dermal papilla as the flame below the kettle since it directs the mitotic activity of the bulb stem cells (18). The cells that form the hair shaft immediately begin differentiation upon release from the germinal bulb matrix. At the electron microscopic level, the high-energy mitotic stem cells of the hair bulb matrix appear the same as primitive epithelial cells, and contain numerous mitochondria (5). Once the daughter stem cells leave the germinal bulb matrix they never divide again, and take on very distinctive roles as differentiated cuticle cells, medullary cells, and cortical cells of the hair shaft (Fig. 2). While other cell deriva-

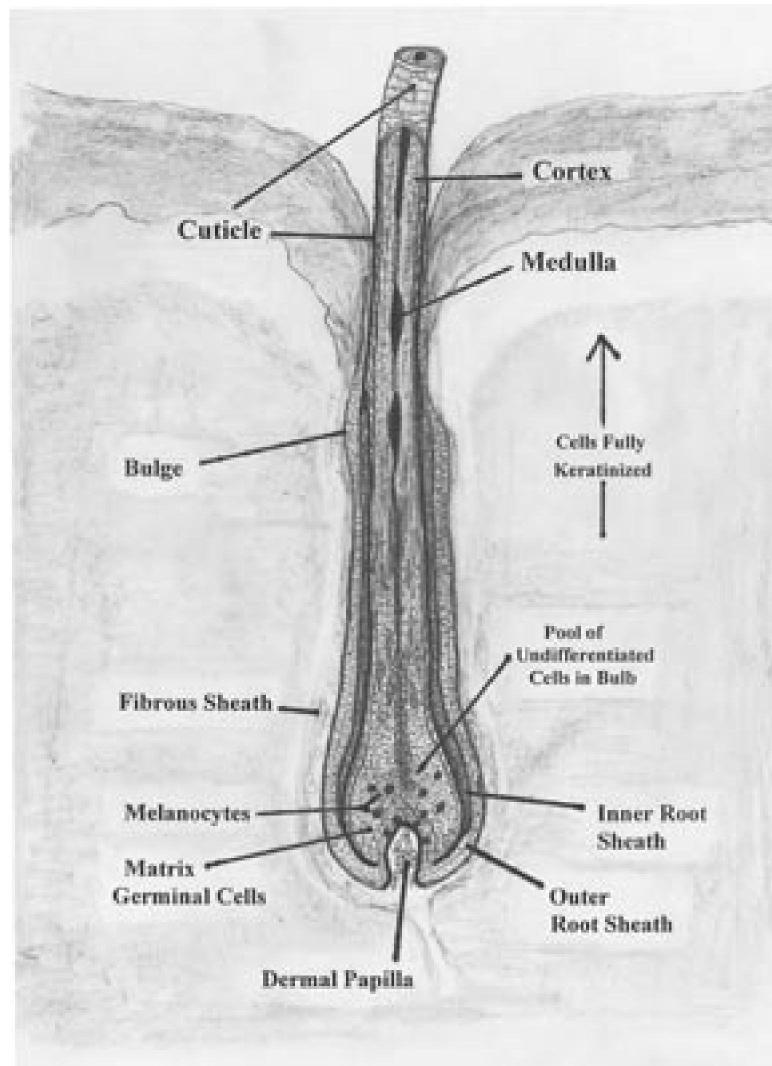

FIG. 2—*Anagen hair root bulb/stem within the follicle (fibrous sheath, inner, and outer root sheath) and emerging hard keratin shaft. All elements below the bulge, together or separate, may be suitable for nucDNA typing.*

tives (which maintain the inner root sheath structures) are produced by the bulb stem cells, the present discussion is limited to a review of histogenic events relevant to mitochondrion found in the keratinized hair shaft.

Hair Melanocytes

The melanocyte, found in the hair root bulb, is a cell with high-energy demands and becomes an old, nonmitotic producer of mitochondria. During fetal development, melanocytic cells are derived from the neural crest of the embryo (i.e., the mesectoderm). These early cells migrate from the neural crest to the developing primitive epidermis, and are randomly distributed. A portion of the active melanocytes become concentrated in the ectodermal portion of the bulbous cell cluster that will ultimately become the hair bulb matrix (Fig. 2) (5). Melanocyte concentrations in the skin vary with the racial origins of individuals. Melanocytes that are present in the hair bulb above the papilla divide slowly, in contrast to the neighboring germinal bulb matrix cells, and remain interspersed in the bulb matrix while the cells that form the hair shaft migrate upward. During maturation, the melanocytes form dendritic arms that contain mitochondria and melanosomes. These dendritic processes are engulfed (via endocytosis) by the passing pre-cortical cells, which move distally away from the immature matrix cells to their ultimate destination in the keratinized hair shaft (19). It is important to note that cortical cells begin intense protein synthesis (i.e., keratin filament production) shortly after differentiation, and would require numerous mitochondria to help facilitate this activity.

Melanocytes produce varying amounts and types of melanosomes (pigment grains) which ultimately determine hair color. Hairs appear colored because the cortical cells have transported the melanin granules produced by the melanocytes from the hair root to the hair shaft. For example, in graying hairs, the melanocytes are few and dormant. In addition, the melanocytes in gray hair root bulbs are large, and have stubby dendrites that are inefficient for melanin granule transfer (20). Cortical cells in the hair shaft (beneath the surface of the cuticle) could carry at least two populations of mitochondria, one population from the germinal bulb matrix cells, and the other from melanocytes. This characteristic immediately allows for a mixing of different pools of mtDNA molecules, having potentially different DNA sequences. Nonetheless, it is unknown as to how many mitochondria are contributed

along with melanin granules to the passing precortical cells by the melanocytes.

As mentioned above, the melanocytes themselves do not become part of the hair shaft, but rather remain at the site of cellular proliferation (i.e., in the hair bulb matrix above the papilla) producing melanosomes and new mitochondria. In addition, melanocytes are potentially the oldest cells continuously contributing mitochondria to the bulk of the hair shaft, as cortical cells have a relatively short nonmitotic life prior to their fate as dehydrated sacs of keratin fibrils. As a result, no significant number of new mitochondria daughter organelles would be expected from the differentiated cortical cells, while new mitochondria would be produced from the older machinery of melanocytes on a continuous basis. Thus, it is possible that the continuous production of new mtDNA molecules allows for genetic drift in the ratio of potential heteroplasmic variants. This is an important characteristic, because it means that a single hair shaft can have varying ratios of heteroplasmic variants along the length of the shaft, or between different hairs originating from the same follicle.

Hair Growth Stages

Hair has a well-defined series of growth, death, and regeneration phases that cycle throughout the lifetime of the individual (Fig. 3). The active hair growth cycle (i.e., hairs in the anagen phase) lasts for as long as 2 to 7 years for human head hairs, and as short as 6 months for eyebrow, limb, and trunk hairs. The shaft of a human head hair increases in length by about 0.35 mm per day, or more than 300 mm in one average growth cycle. Bone marrow is the only other normal tissue with such a high rate of mitotic activity (21,22). Differences in lengths of hair shafts produced each day are not due to differences in replacement rates of individual germ cells, but are merely a reflection of the size of the germ population, all of which are reproducing at the same rate (5). In addition, there is a direct correlation between root bulb size and hair shaft diameter size.

After this period of programmed rapid growth, the hair ceases to grow, signaling the catagen phase. The catagen, or regression phase, is established by termination of mitosis in the hair bulb matrix cells, and lasts several weeks. This brief period of existence explains why catagen hairs are seldom found as evidence at crime scenes. The melanocytes in the hair bulb matrix become dormant prior to the catagen stage, and thus, melanogenesis ceases prior to the last mitotic divisions of the stem cells; resting, or telogen hairs usually show no evidence of pigmentation in the basal portion of an otherwise heavily pigmented hair shaft. The hair root stem shrinks and retracts upwards through an apoptotic process toward the hair bulge, and the external root sheath shrinks, leaving behind a fibrotic streamer (Figs. 2,3) (23). These events mark the beginning of the telogen (resting) phase. As a result, a telogen hair root is about $1/3$ as long as a root in the anagen phase, and therefore $1/3$ as deep in the skin. In early telogen phase, rootlets from the club anchor into the surrounding soft tissue. Such an arrangement allows for the common forensic finding of follicular tissue on a telogen club that is sometimes suitable for nucDNA typing. In fact, this is the most common root end morphology found in pubic hairs recovered from crime scenes since they spend more time in the telogen phase than the anagen phase.

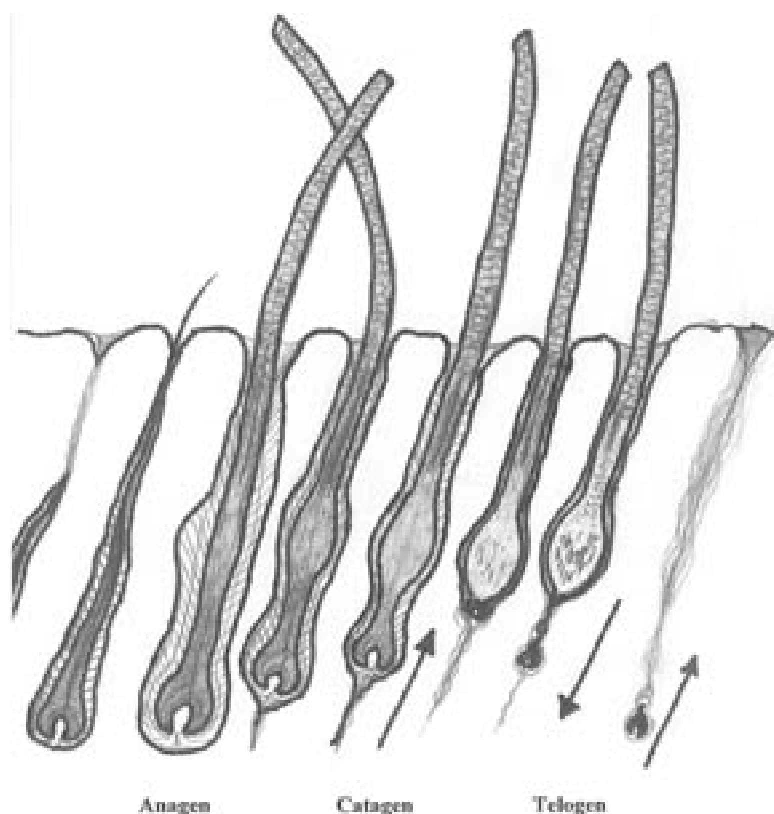

FIG. 3—*Human hair growth, degeneration, and regeneration stages. Far left, new growth anagen hair. Far right, descended telogen germinal unit (follicle bud) ready to begin new hair growth (proanagen). The stem cells for this new growth are thought to have origin from the hair root bulge. This process takes 2 to 7 years for head hairs and only about 1 year for pubic hairs.*

Some have suggested that the mitochondrion is an agent of programmed cell death in other tissues (24), however, its role in the apoptosis of the hair root is unknown. Nonetheless, in the telogen phase, the lower end of the hair root stem finally comes to rest as a club or resting hair at the level of the hair root bulge, surrounded by a sac of keratin. The shrunken dermal papilla is then dragged upwards beneath a newly formed telogen germinal unit. Reports suggest that the new stem cells of the telogen germinal unit have their origin from the hair root bulge, or that area where the arrector pili muscle inserts into the hair follicle (17) (Fig. 2). A new follicle bud (i.e., germinal unit of stem cells and shrunken dermal papilla) develops below the hair root club during the approximate three month duration of the telogen phase. This follicle bud ultimately descends from beneath the dead telogen root club and reverts to the subcutaneous tissue to develop a new anagen hair (i.e., the proanagen phase).

Finally, a new anagen phase begins when growth factors from the descended dermal papilla directs the unorganized group of epithelial cells comprising the germinal unit above it to produce the new hair. When telogen follicles become anagen again, the sequence of events is similar to that of the original morphogenesis in fetal skin, as this process 'restarts' with the same basic machinery present in the previously lost hair. In addition, the telogen hair is usually shed after the new anagen hair begins to grow beneath it, thus ending one hair growth cycle, and beginning another. In essence, the follicle bud which produces the new anagen hair originates from the previous telogen hair, which was shed from the same site in the skin.

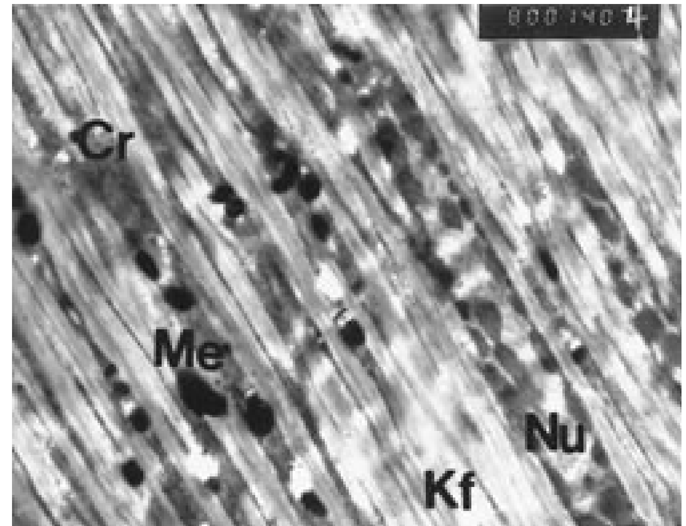

FIG. 4—*Caucasoid brown head hair shaft above the level of the hair root bulb. Cortical cells nearing complete cytolysis. Degenerating nucleus (Nu), keratin filament bundles (Kf), melanin granules (Me), and cytoplasmic remnants (Cr). Transmission electron microscopy. Uranyl acetate and lead citrate staining. (Original magnification, ×8000.)*

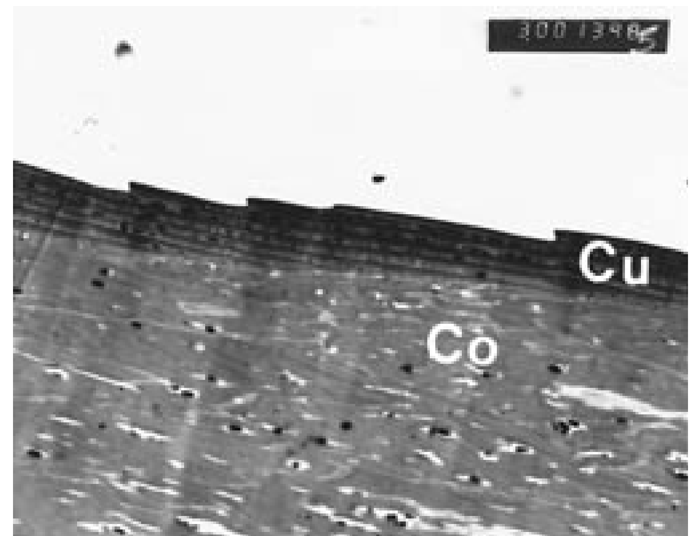

FIG. 5—*Caucasoid brown head hair shaft with completely keratinized cuticle (Cu) and cortex (Co). Note cytoplasmic debris shifted to inner side of each overlapping cuticle plate (endocuticle). The interlocking dead cortical cells below the cuticle show prominent melanin granules. The spindle-shaped voids are cortical fusi. Transmission electron microscopy. Uranyl acetate and lead citrate staining. (Original magnification, ×3000.)*

Hair Shaft Cellular Differentiation and Disintegration

The keratinized shaft of the human hair is the component of the hair that most frequently requires mtDNA analysis in criminal cases. The sources of these keratinized segments are hairs with telogen club roots absent soft tissue, hair fragments without roots, or hairs with root soft tissue that failed to produce sufficient nucDNA results. The fully keratinized region of the hair begins above the level of the hair root bulb (Fig. 2), and the mature shaft is composed of a cuticle, cortex, and medulla. For illustration purposes, the human hair is commonly described as a wooden pencil. (This analogy has been used frequently to explain hair structure to juries.) The yellow paint coating of the pencil corresponds to the hair cuticle, with the wood being the hair cortex, and the graphite core being the hair medulla. These structures are easily identified by transmitted light microscopy. However, no cell architecture is visible by transmission or scanning electron microscopy since the cells of the mature shaft are dead and composed almost entirely of tightly packed keratin fibrils embedded in an amorphous matrix of cellular debris (5) (Fig. 4). Thus, the majority of the mature hair shaft is composed of structural protein fibrils belonging to four multigene families (22).

The hair cortex comprises the bulk of the hair shaft (Fig. 5). Precortical cells, or keratinocytes in the hair root bulb begin intense protein synthesis with the appearance of keratin tonofilaments in their cytoplasm shortly after differentiation. Numerous mitochondria can be seen in these young high-energy cells (5,25). As mentioned previously, the pre-cortical cells are also the primary recipients of the melanin granules and perhaps mitochondria from the stationary melanocytes before being pushed upward by the constant cell proliferation below. The color, size, distribution, and overall pattern of these pigment grains is probably the most important microscopic feature observed and compared by hair examiners. Premature cortical cells connect to one another by desmosomes and gap junctions as they rise and mature in the hair root stem. Cortical cells continue their migration upward and increase keratin fibril production until their cytoplasm is eventually filled with thick fiber bundles. Upon leaving the prekeratinous zone of the root stem and making entry into the keratinous zone of the hair shaft (above the root bulge), the cortical cell's diameter decreases by about 25% due to dehydration and contraction of the protein fibril complexes in the cytoplasm. At the point of complete keratinization, cytolysis occurs with nuclei losing their DNA, and mitochondria and ribosomes beginning to disintegrate. Nonetheless, mitochondria can be

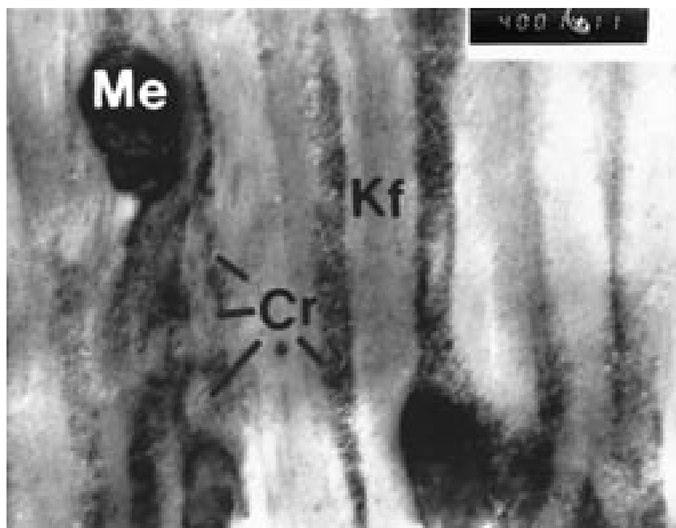

FIG. 6—*Caucasoid brown head hair shaft above the level of the hair root bulb. Ovoid cytolytic remnants have been crowded together between keratin filament bundles (Kf). Cytoplasmic remnants, left, mitochondria (Cr), and melanin granule (Me). Transmission electron microscopy. Uranyl acetate and lead citrate staining. (Original magnification, ×40 000.)*

seen crowded between the thick filament bundles in the keratinizing hair cortex cell (Fig. 6). The dead cortical cells retain a membranous nuclear outline (i.e., nuclear ghost) that persists into the hair shaft. Presumably, the mitochondrion offers protection for the mtDNA molecules, which is unavailable to nucDNA during the early stages of gradual cellular cytolysis.

Cuticle cells also originate from the hair bulb matrix, but differentiate into structures very dissimilar from the cortical cells. Cuticle cells elongate and flatten in the suprabulbar region of the hair stem, migrate laterally, and ultimately emerge as hard keratin overlapping flat plates, or scales, on the surface of the hair shaft (25). Each plate represents one cell. In addition, during keratinization of the cuticle cells, trichohyalin granules and debris from the nucleus, ribosomes, and mitochondria are shifted to the inner side of the cytoplasm and form an endocuticle for each cuticle plate (Fig. 5).

Medullary cells are the final contributors of the hair shaft. These cells do not produce as much structural protein as cuticle cells or cortical cells. Medullary precursor cells are the most centrally located within the concentric ring of germ cells above the dermal papilla. In the final stages of differentiation, and similar to the cuticle and cortical cells, the organelles and nuclei of medullary cells begin to disintegrate. Some of these loosely packed cells disintegrate rapidly with evidence of cytoplasmic vacuolation occurring as soon as they reach the central core of the hair shaft bulb.

The microscopic appearance of the medulla is another important comparison feature used by hair examiners. Hair examiners state that a hair medulla is "present" if they see a continuous or fragmented blackened structure in the center of a hair shaft (Fig. 2). Air spaces in hair appear black in resin (e.g., Permount) embedded microscope slide preparations. This black linear structure, which occupies less than one third of the total hair shaft diameter in humans, is actually an open column of air in the center of the hair shaft where the loosely packed medullary cells have completely disintegrated. Thus, when hair examiners state that a medulla is "absent," this central space in the shaft is filled with fully keratinized medullary cell debris or cortical cells. While medullas seem to appear more frequently in coarse hairs (at least in humans), an individual's scalp hair may show a variety of medullated, minimally medullated, and nonmedullated hairs. Nonetheless, hair shafts with medullas present would be expected to contain less mitochondrial debris, and subsequently less mtDNA than hair shafts without a medulla.

Discussion

The first forensic case where mtDNA results were introduced into a court of law in the United States was the *State of Tennessee* vs *Paul William Ware* in September of 1996 (an FBI Laboratory case). This case involved analysis of a red Caucasoid pubic hair found in the throat of a four-year-old female child who was sexually assaulted and murdered. The mtDNA profile of the hair matched Mr. Ware's profile, and thus, he could not be excluded as the perpetrator. In combination with other investigative evidence, Mr. Ware was convicted of the crimes. In all, mtDNA analysis has been used in more than one thousand forensic cases worldwide; although very few have been published (26). In the past four years alone, mtDNA results have been admitted into evidence in more than fifteen states in the United States (including Indiana, Maryland, Michigan, New Mexico, North Carolina, Pennsylvania, South Carolina, Tennessee, Texas, Virginia, and Washington).

With the growing number of laboratories worldwide that are beginning to use mtDNA sequence analysis in routine forensic casework, a greater understanding of human hair histogenesis may enhance the forensic scientist's ability to relate the physical characteristics of a hair to observed mtDNA sequence information. For example, primordial germ cells, as found in the hair bulb matrix, each contain about 1000 mtDNA molecules (22,25). These are the mtDNA precursor molecules that the hair will have throughout the lifetime of the individual, without introduction from external factors, and from which future mtDNA molecules will be replicated. Thus, the question that immediately arises in a forensic context is—What effect, if any, does the clonal nature of each hair have on how mtDNA sequence data is interpreted?

In the past, the composition of mtDNA in the cells of an individual (including primordial germ cells found in hairs) was considered to be homoplasmic for a single mtDNA sequence or haplotype. However, it is now commonly accepted that heteroplasmy is present to some degree in all individuals; most individuals possess very low levels of heteroplasmic variants undetectable by DNA sequence analysis. In practice, levels of heteroplasmy in peripheral blood can be detected by DNA sequence analysis if the minor component is greater than 10 to 20% of the overall mixture. In doing so, detectable levels of heteroplasmy have been observed in approximately 5% of the individuals analyzed.

When heteroplasmy is observed in the blood of an individual, this immediately suggests that heteroplasmy will be observed in representative hairs of that same individual. On the other hand, while minor heteroplasmic components of an apparently homoplasmic individual are generally undetectable without using a more sensitive detection method (e.g., cloning or denaturing gradient gel electrophoresis), it is important for the mtDNA scientist to consider that undetectable heteroplasmic variants can become major components of an individual's haplotype through the action of genetic bottlenecks (e.g., cellular replication, segregation, or differentiation). Since the cells feeding the hair shaft have a restricted or clonal set of mtDNA molecules (acting as a genetic bottleneck), it is very likely that differing subpopulations of heteroplasmic variants may be present in the scalp of each individual. As a result,

mtDNA haplotypes of different hairs analyzed from the same individual may have consistent levels of a single heteroplasmic variant, may have a variety of heteroplasmic variants, or may have apparent substitution events from one major sequence haplotype to another. In addition, this phenomenon is not restricted to apparent homoplasmic individuals, as this can also occur in individuals who have detectable levels of heteroplasmic variants. Thus, in a forensic context the question that immediately arises is—How would this characteristic of human hairs be revealed in actual casework?

The human scalp contains about 100 000 to 150 000 hair follicles containing distinct populations of cells, and thus, discrete subpopulations of mtDNA molecules. Roughly 10% of these hairs are in the telogen phase (which lasts about three months) at any one time in the nonbalding scalp, so approximately 150 head hairs can be shed every day from a single individual. While none of these telogen club hairs are suitable for nucDNA typing, most are suitable for mtDNA sequence analysis. Taking a step back, if each cell in the hair root bulb contains about 1000 copies of mtDNA, and there are around ten stem cells (very conservative estimate) that make up a thin hair root bulb matrix, there could be 1 000 000 000 to 1 500 000 000 initial copies of mtDNA in the human scalp. If we restrict our analogy to these initial copies of mtDNA, there would be roughly 1 500 000 copies of mtDNA which are shed daily from a single individual. The genetic bottleneck created by the clonal nature of each of these shed hairs not only allows for significant disparity in the initial ratio of any heteroplasmic variants (minor or major), but also helps to facilitate fluctuation in the ratios of these variants throughout the lifetime of an individual, or even the lifetime of an individual hair. For example, in a population of 150 shed hairs or 1 500 000 mtDNA molecules, there could be a small sub-population of approximately 150 000 molecules with a different mtDNA sequence. This minor variant could be distributed evenly (approximately 10% in each hair), or could be present in differing ratios of major and minor variants across each hair. The more likely outcome, of course, is the latter possibility. Thus, it is likely that a small number of hairs will exhibit higher levels of the minor variant, some to the point where the minor variant becomes the major component. In some instances, this could result in an apparent shift in haplotypes from one apparent homoplasmic sequence to another. Thus, if 20 hairs from an individual exhibiting these characteristics were analyzed, one might expect to observe about ten hairs with the major haplotype, roughly eight hairs with a mixture of the two haplotypes (i.e., heteroplasmy), and one or two hairs that are an apparent shift to the haplotype of the minor component.

So why don't we all have high levels of multiple heteroplasmic variants? The answer is quite complex, and is not a completely understood process. During development of the human oocyte, the number of mtDNA molecules is reduced in size to a very small number (some estimates are less than ten copies (1)). This condensed population generally reflects the major mtDNA haplotype, and excludes the potential multitude of minor variants. Nonetheless, why don't some of these "micro-variants" slip through the bottleneck? The answer may be that the reduction in mtDNA copy number occurs during fetal development (i.e., primordial oocyte development in females that is completed before birth), and thus, the number of accumulated micro-variants (due to somatic mutation) will be very small. While somatic mutations may cause mtDNA variants to accumulate in adult tissues (10), these variants have little effect on reproductive cells, and do not appear to effect the haplotype of future offspring. As a result, individuals generally inherit only one major heteroplasmic variant, if any, because the action of intergenerational genetic bottlenecks maintains the number of variants to a small number (Fig. 7).

In summary, variation in mtDNA sequence heteroplasmy in human hairs can generally be attributed to genetic bottlenecks that occur during transmission of mtDNA from mother to child, during development of the fetus (including cellular differentiation), and even through the simple process of cell division. Given what we know about the embryology, differentiation, and characteristics of the cells that constitute the hair follicle and shaft, it is now clear that

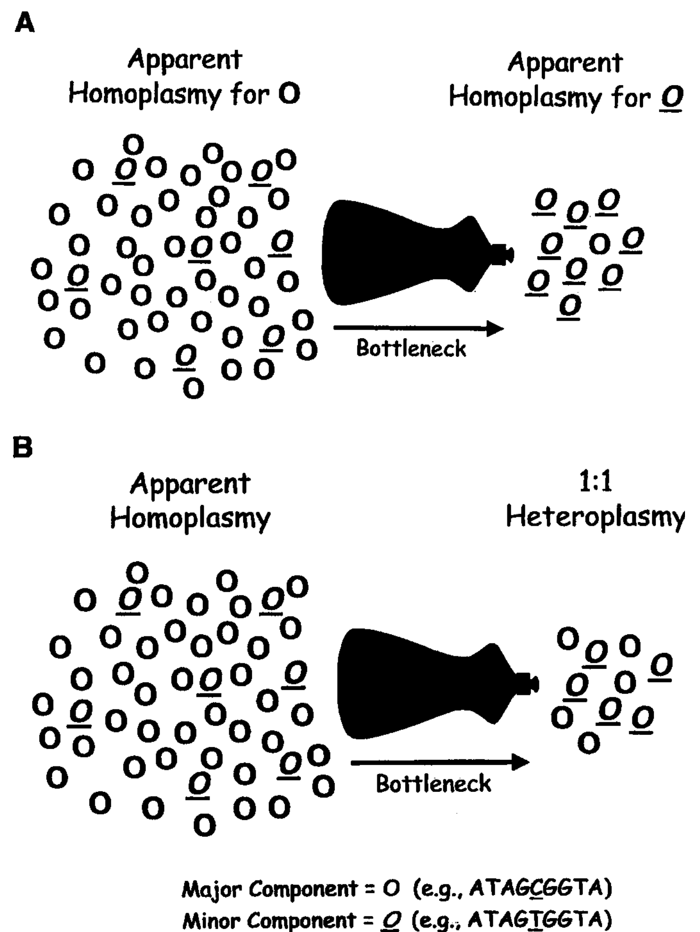

FIG. 7—*The bottleneck theory of mtDNA segregation involves a reduction in the number of copies of mtDNA at the primordial germ cell stage of the immature mammalian oocyte (30). The composition of mtDNA sequences that exist in the primordial germ cell may vary, to include different ratios of heteroplasmic variants. During the reduction process, copies of mtDNA are presumably chosen at random, resulting in a variety of possible combinations of major and minor sequence components. Panel A illustrates how an mtDNA profile of apparent homoplasmy can shift in one generation to a different, apparently homoplasmic sequence. It is important to note that, in general, a single sequence difference will be observed across 610 (or more) nucleotide positions. In this case, the sequence difference is an apparent C to T transition. Panel B illustrates that detectable heteroplasmy could be observed in this same individual, to include an even distribution of the previously major and minor variant sequences. When performing DNA sequence analysis, these distributions can usually be detected when the ratio of the major to minor variants exceeds a 10:1 to 5:1 ratio. The principles illustrated in Panels A and B of this figure can be applied to the segregation processes that occur in the human hair. For example, isolated populations of cells will segregate from one another as hair follicles are being established during early fetal development. As a result, these remote clusters of cells may have different ratios of heteroplasmic variants, or could even possess mtDNA molecules that have somatic mutations.*

hairs from the same scalp of an individual with known heteroplasmy, or even from an individual with undetectable levels of heteroplasmy, may show different mtDNA sequences from one another. Whether through inheritance or somatic mosaicism, each hair follicle contains a discrete group of stem cells that will represent a subset of this heteroplasmy throughout the lifetime of the individual. The averaging of haplotypes from numerous different hair stem cell populations is not possible in forensic single hair casework samples, as what routinely occurs in forensic peripheral blood analysis (27). Thus, in order to further investigate the occurrence of mtDNA sequence heteroplasmy in single human hairs, and to recognize those hair types that are difficult to sequence, investigators may consider recording the following data prior to analysis, when possible.

- Is the hair heavily pigmented and what hues are present? In other words, does the melanocytic mitochondrial contribution versus the keratinocyte contribution have any relation to the incidence of observed heteroplasmy? The recording of racial information is important, but information regarding heavy pigmentation may be lost in simple Caucasoid, Mongoloid, or Negroid group listings. Microscopic pigment color(s), pigment grain size/distribution, and degree of clumping may be useful to include in such studies; these categories should be in addition to those that catalog artificial hue or natural hue. Grey hairs would not be expected to contain mitochondria with melanocytic origin since transferal of melanin grains and mitochondria from the melanocyte to the hair shaft occurs in the same event. Photomicroscopy of hair shaft sections prior to analysis would be the best documentation of these and other internal microscopic characteristics. There have been no reports in the literature which relate degree of hair pigmentation to observed mtDNA heteroplasmy.
- Which section of hair shaft did the sample come from (if this information is available)? Distal segments of hair shaft could experience greater environmental effects (weathering, chemical) than proximal segments. Distal hair segments are products of anagen cellular machinery while proximal segments are potential products of catagen or telogen cellular machinery. A telogen root club and the immediate adjacent hair shaft would not be expected to contain mitochondria from melanocytes since they become dormant prior to the telogen stage.
- Is root follicular tissue present versus strictly keratinized hair shaft? Mitochondrial populations from fresh soft tissue and mitochondrial populations from keratin embedded hair shafts may or may not show differences. Mesoderm (dermal papilla) and endoderm (hair root bulb) germ cells in the growing hair root may have differing ratios of heteroplasmic variants. Biomedical investigators more frequently report mtDNA analysis of soft tissue specimens from hair roots, rather than hair shafts, as reported by forensic investigators.
- What is the age of the donor? Does hair mtDNA sequence heteroplasmy increase with donor age? This has been suggested from other studies that have included portions of the mtDNA control region (28). Since there are no additional hair follicles that are formed after birth, donor age could have a substantial effect. In addition, the melanocyte is a cell with high-energy demands and becomes an old, nonmitotic producer of mitochondria. Human brain cells are nonmitotic cells with a high-energy demand and a long life. They have been reported to have high levels of mtDNA sequence heteroplasmy in the noncoding control region (29).
- What is the diameter of the hair shaft and what cross sectional shape does the hair have? Head hairs from the same person will vary in diameter so that a 2 cm piece of one head hair shaft may contain many more, or less, mitochondria than another 2 cm piece of head hair shaft from the same scalp. Human head hair shaft diameters range from 60 to 84 μm and a single hair shaft can exhibit highly variable diameters at different points along the shaft (5). Persons of strictly Asian descent have head hairs with round cross-sectional shapes, while persons of strictly African descent have head hairs with flat cross-sectional shapes. Pubic hairs in all racial groups can exhibit a flat cross section and often have a "trough" running down the center surface of one flat side. There have been no reports in the literature which relate hair shaft size to observed mtDNA heteroplasmy.
- Is a medulla present or absent? The answer to this question may be useful to document when evaluating the success rate when sequencing different types of hairs. Hairs with continuous, broad medullas would be expected to have less mitochondrial debris, and therefore less mtDNA molecules, than hairs without a visible medulla. Facial hairs can have very broad medullas.
- What is the somatic origin of the hair? Hairs have much different periods of growth depending upon which part of the body they came from. One might expect more chance for change in mitochondrial populations in head hairs than in pubic hairs due to the difference in number and length of germ cell cycles for each region. Head hairs grow two to seven years and rest for about three months while pubic hairs grow for about six months and rest for six or more months. Pubic hairs, on the other hand, have larger root bulbs, and subsequently larger germ cell and mitochondrial populations.
- Is there any sign of hair disease? Pili annulati is characterized by periods of normal keratin formation followed by intermittent periods of defective keratin formation. Visual inspection of the hair reveals a banding pattern of colors. This relatively rare condition and other hair disorders are confirmed by light microscopy and the patient recognizes no physical discomfort. However, the possible effects of disease have not been associated with mtDNA sequence analysis of hair shafts.

Answers to these questions are not consistently available in the literature and further research and data collection is needed to determine if any of these issues are relevant to observations of mtDNA heteroplasmy in hairs. The need for careful microscopic identification and comparison of hairs prior to mtDNA analysis, to eliminate those hairs which are not probative to an investigation, is even greater than that for nucDNA analysis because mtDNA analysis is considerably more costly and time consuming. There is also a need to attempt to identify those hair types which may be the best candidates for mtDNA analysis in the event several hairs in a case become candidates for this analysis as determined through comparison microscopy. As forensic scientists generate greater amounts of mtDNA sequence data, we can begin to try and answer many of the questions concerning the relationship of hair histogenesis and mtDNA analysis. Until then, the exploitation of the naturally occurring variation in human hairs, by both the microscopist and the DNA analyst, continues to contribute to the search for truth in forensic science.

References

1. Holland MM, Parsons TJ. Mitochondrial DNA sequence analysis—validation and use for forensic casework. Forensic Sci Rev 1999;11:21–50.
2. Budowle B, Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM. Population data on the thirteen CODIS core short tandem repeat loci in African Americans, U.S. Caucasians, Hispanics, Bahamians, Jamaicans, and Trinidadians. J Forensic Sci 1999;44(6):1277–86.
3. Linch CA, Smith SL, Prahlow JA. Evaluation of the human hair root for DNA typing subsequent to microscopic comparison. J Forensic Science 1998;43(2):305–14.
4. Walsh PS, Varlaro J, Reynolds R. A rapid chemiluminescent method for quantitation of human DNA. Nucleic Acids Res 1992:20(19):5061–5.
5. Olsen EA, editor. Disorders of hair growth, diagnosis and treatment. Chapter 2: Differentiation of the hair shaft. New York: McGraw-Hill Inc., 1994.
6. Michaels GS, Hauswirth WW, Laipis PJ. Mitochondrial DNA copy number in bovine oocytes and somatic cells. Dev Biol 1982;94(1): 246–51.
7. Robin ED, Wong R. Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. J Cell Phys 1988;136: 507–13.
8. Bisbing RE. The forensic identification and association of human hair. In: Saferstein R, editor. Forensic Science Handbook. Englewood Cliffs, NJ: Prentice Hall, 1982.
9. Smith SL, Linch CA. A review of major factors contributing to errors in human hair association by microscopy. Am J Forensic Med Pathol 1999;20(3):269–73.
10. Tully LA, Parsons TJ, Steighner RJ, Holland MM, Marino MA, Prenger VL. A sensitive denaturing gradient-gel electrophoresis assay reveals a high frequency of heteroplasmy in hypervariable region 1 of the human mtDNA control region. Am J Hum Genet 2000;67(2):432–43.
11. Parsons TJ, Muniec DS, Sullivan K, Woodyatt N, Alliston-Greiner, Wilson MR, et al. A high observed substitution rate in the human mitochondrial DNA control region. Nat Genet 1997;15:363–8.
12. Awadalla P, Eyre-Walker A, Smith JM. Linkage disequilibrium and recombination in hominid mitochondrial DNA. Science 1999;286:2524–5.
13. Eyre-Walker A, Smith NH, Smith JM. How clonal are human mitochondria? Proc R Soc Lond B 1999;266:477–83.
14. Holt IJ, Harding AE, Morgan-Hughes JA. Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature 1988;331(6158):717–9.
15. Gill P, Ivanov PL, Kimpton C, Piercy R, Benson N, Tully G, Evett I, Hagelberg E, Sullivan K. Identification of the remains of the Romanov family by DNA analysis. Nat Genet 1994;6:130–5.
16. Wilson MR, Polansky D, Repogle J, DiZinno JA, Budowle B. A family exhibiting heteroplasmy in the human mitochondrial DNA control region reveals both somatic mosaicism and pronounced segregation of mitotypes. Hum Genet 1997;100:167–71.
17. Paus R, Cotsarelis G. The biology of hair follicles. N Engl J Med 1999; 341(7):491–7.
18. Lyne AG, Short BF, editors. Biology of the skin and hair growth. New York: American Elsevier, 1965.
19. Birbeck MSC, Mercer EH, Barnicott NA. The structure and formation of pigment granules in human hair. Experimental Cell Research 1956;10:505–14.
20. Nordlund JJ, Boissy RE, editors. The pigmentary system, physiology and pathophysiology. Oxford: Oxford University Press, 1998.
21. Lever WF, Schaumberg-Lever G, editors. Histopathology of the skin. Chapter 3: Histology of the skin. Philadelphia: J.B. Lippincott Co., 1983.
22. Ackerman AB, DeViragh PA, Chongchitnant N. Neoplasms with follicular differentiation. Chapter 4: Anatomic, histologic, and biologic aspects of hair follicles and hairs. Philadelphia: Lea and Febiger, 1993.
23. Weedon D, Strutton G. Apoptosis as the mechanism of the involution of hair follicles in catagen transformation. Acta Dermatovener 1981;61: 335–69.
24. Singh KK, editor. Mitochondrial DNA mutations in aging, disease, and cancer. Berlin: Springer-Verlag, 1998.
25. Toda K, Ishibashi Y, Hori Y, Morikawa F, editors. Biology and disease of the hair. Baltimore: University Park Press, 1975.
26. Allen M, Engstrom AS, Meyers S, Handt O, Saldeen T, von Haeseler A, et al. Mitochondrial DNA sequencing of shed hairs and saliva on robbery caps: sensitivity and matching probabilities. J Forensic Sci 1998;43(3): 453–64.
27. Bendall KE, Macaulay VA, Sykes BC. Variable levels of a heteroplasmic point mutation in individual hair roots. Am J Hum Genet 1997;61:1303–8.
28. Calloway CD, Reynolds RL, Herrin Jr GL, Anderson WW. The frequency of heteroplasmy in the HVII region of mtDNA differs across tissue types and increases with age. Am J Hum Genet 2000;66(4):1384–97.
29. Jazin EE, Cavelier L, Eriksson I, Oreland L, Gyllensten U. Human brain contains high levels of heteroplasmy in the noncoding regions of mitochondrial DNA. Proc Natl Acad Sci USA 1996;93:12382–7.
30. Jenuth JP, Peterson AC, Fu K, Shoubridge EA. Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. Nat Genet 1996;14:146–51.

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