MILLIGAN’S TRICHOROME STAIN FOR USE IN COMPARING THE DEVELOPMENT OF PENILE SPINES TO HAIR AND NAILS IN MICE

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ABSTRACT

Penile spines are prevalent throughout the primate world, however they are absent in humans. Penile spines are keratinized, androgen dependent structures that protrude from the glans of the penis. The lack of penile spines has been linked to a longer time of intromission during copulation and is hypothesized to be part of an adaptive suite linked to pair-bonding in humanity’s early hominid ancestors. Other appendages of the skin, such as hair, nails, mammary glands, and teeth all show similar developmental origins through specific interactions between the epithelium and mesenchymal tissue. The purpose of this study is to determine the efficacy of Milligan’s trichrome stain for distinguishing between muscle, collagen, and keratin during skin appendage development. Once this was established, the histology of penile spine, hair, and nail development was compared during postnatal mouse development. Milligan’s trichrome stain proves to be effective for distinguishing keratinized structures and may even be useful in distinguishing between hard and soft keratins. In agreement with previous studies on skin appendage development, penile spine formation is shown to be largely similar to that of hair and nails. However, penile spines develop substantially later than these other structures, with their entire development occurring postnatally. Placodes appear at P7, buds appear by P15, and the keratinized spines appear at P20. By P63 they are fully formed keratinized spines that are exposed to the exterior.
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Chapter 1
Introduction

Human Distribution of Androgen Receptor

Humans possess very peculiar external phenotypes. Specifically, we have a distinct set of secondary sexual characteristics compared to our primate relatives. The male-specific secondary sex characteristics result from tissue specific responses to circulating androgens (testosterone and dihydrotestosterone). These tissue specific responses are mediated, in part, by androgens binding to Androgen Receptor (AR). Thus, mechanisms likely exist to generate a species-specific distribution of AR throughout the body. These responses have been studied extensively and include male sexual differentiation, growth, and maintenance of male secondary sex characteristics\(^1\).

Testosterone has different phenotypic effects in individual tissues, which can largely be attributed to AR. One example of this in humans is the android and gynoid adipose distribution patterns. These patterns arise because AR is differentially expressed between intra-abdominal and subcutaneous preadipoctyes\(^2\). AR also plays a key role in the development of many types of skin appendages, including: teeth\(^3\), mammary glands\(^4\), feathers\(^5\), hair\(^6\), and penile spines\(^7\). Each of these skin appendages develops via a similar mechanism of epithelial-mesenchymal interactions. AR also plays a role in the epithelial-mesenchymal interaction in urogenital development\(^8\).

Many mammals form keratinous protrusions in the epidermis of their penises known as penile spines. These develop as a local thickening of the epithelium\(^9,10\). Primates are one of a number of mammalian orders that have penile spines. Studies in the male greater galago (Galago crassicaudatus) have shown that castration, and thus a loss of testosterone production, results in
the failure of spine formation on their penises. In addition, treatment with exogenous doses of testosterone leads to the rescue of penile spines\textsuperscript{11}. Knockout mutations of $AR$ in mice have also shown a subsequent lack of penile spines\textsuperscript{12}. This demonstrates that both $AR$ and testosterone play key roles in the formation and maintenance of penile spines.

Humans, of course, are one of the exceptions in the primate order that do not possess penile spines. Humans are missing a conserved enhancer sequence that appears to direct $AR$'s spatial expression\textsuperscript{13}. This sequence is a 5 kb non-coding sequence that is conserved in many mammals, including chimpanzees and mice. In chimps, this sequence is contained in a 60.7 kb sequence between the $AR$ and the \textit{Oligophrenin 1} (OPHN1) loci. This entire 60.7 kb sequence has been evolutionarily deleted in humans. To further determine the significance of this deletion to humans, 4.8 kb of non-coding sequence that is highly conserved between chimpanzees, macaques, and mice was cloned from the chimpanzee and placed in a vector containing a basal promoter driving $\text{lacZ}$ gene expression. When inserted into mouse oocytes, the reporter gene was observed in the genital tubercle, facial vibrissae, mammary glands, and hair follicles at embryonic day (E) 16.5\textsuperscript{14}. Specifically in the penis, the reporter sequence was seen in the superficial mesoderm within the presumptive glans of the developing genital tubercle\textsuperscript{7}. This indicates that the sequence functions as an enhancer during the development of some skin appendages.

The loss of the conserved enhancer sequence is a potential genetic and developmental mechanism underlying the lack of penile spines in humans. Other experiments have shown that ablation of the penile spines in marmosets results in a significant increase in intromission duration during copulation\textsuperscript{10}. McLean et al. hypothesize that the genomic deletion and associated loss of penile spines as one potential mechanism underlying the longer human copulation duration compared to chimpanzees. These traits, along with other deviations from chimpanzees, such as feminization of the male canine dentition, moderate testes size, low sperm motility, and enlarged
mammary glands, may have arisen as a result of an increase in pair-bonding and parental care in humans\textsuperscript{15}.

**Overview of Skin Appendage Morphogenesis**

The integument system is composed of several layers. These include, from most superficial to deep, the epidermis, the dermis, and a layer of adipose tissue called the hypodermis. The epidermis covers most of the body and is composed of multiple layers. Its main role is to protect against foreign pathogens and prevent essential fluid loss\textsuperscript{16}. In the developing embryo, the ectoderm differentiates into either nervous tissue or skin epithelium following gastrulation. Wnt signaling blocks Fibroblast Growth Factor (FGF) signaling, allowing Bone Morphogenetic Proteins (BMPs) to signal cells to form the epidermis. Nervous tissue arises in an absence of Wnt signaling, resulting in the expression of FGFs and inhibition of BMP signaling\textsuperscript{17}. Initially, embryonic epidermis consists of only one layer of multipotent epithelial cells. These multipotent cells then stratify and differentiate into the various layers of the epidermis (Figure 1-1)\textsuperscript{16}.

Later in development, various skin appendages, such as hair, teeth, mammary glands, and

![Figure 1-1. Early signaling in Ectodermal differentiation during Gastrulation](image)

Without Wnt signaling, FGF is expressed and inhibits BMP signaling which fates cells to become neural progenitor cells. When Wnt is present, it inhibits FGF signaling which in turn causes BMP to fate cells to become epidermis. The epidermis differentiates to become either stratified squamous epidermis or hair cells as it continues to respond to Wnt from the epidermis and BMP, EGF, and Notch in the mesenchyme. Adapted from [16]
nails begin to form. Each of these appendages initially arises through the interactions between epithelial and mesenchymal layers of the skin. At specific locations the epithelium thickens to form a placode that subsequently invades the presumptive dermis to form a bud. At these early stages, the development of these various structures depends on many of the same genes and transcription factors. The signaling of a few genes plays a particularly important role in these initial epithelial-mesenchymal interactions during the formation of each of these appendages including: Wnt, FGFs, transforming growth factor beta (TGFβ), and Sonic hedgehog (Shh). In addition, these processes appear to be highly conserved across mammalian species. The depth of similarity is illustrated by recent experiments in which dental papillae mesenchymal cells are capable of inducing skin epithelial cells to become ameloblasts that can ultimately give rise to new teeth. However, after bud formation, the morphogenesis of these different skin appendages begins to diverge, with each undergoing specific patterns of tissue folding and growth.

**Hair Development**

One of the most studied skin appendage is hair. Dermomyotome, a precursor to both the dermis and skeletal muscle, begins the process by differentiating into mesenchymal cells through Wnt signaling. As mesenchymal cells populate and spread out under the skin, a placode results from the thickening of epidermal keratinocytes. Epithelial-mesenchymal tissue recombination experiments in mice have revealed that signals from the mesenchyme are responsible for the positioning and distribution of the placodes. Both FGF signaling and BMP inhibiting factors are crucial for inducing placode formation. In mice, placode formation is thought to occur in three consecutive waves corresponding to the three types of murine hair. The first wave consists of guard hairs, followed by a wave of awl/auchene hairs, and lastly the zig zag hairs. The primary
hair placodes first appear around embryonic day 13.5 (E13.5). The second wave begins around E16 and the third wave begins at E18\textsuperscript{18}.

Wnt signals become an important factor for initiating downward growth of the placode to form the hair bud. Shh functions to organize mesenchymally derived cells into the dermal papilla, which ultimately becomes part of the hair follicle. The dermal papilla then signals back to the placode to initiate its down growth into the dermis\textsuperscript{23}. Additional reciprocal signaling between the epithelium and mesenchyme are required for continued maturation of the hair follicle\textsuperscript{16}. Placodes themselves also produce Wnt inhibitory factors and BMPs that contribute to a negative feedback loop preventing other placodes from forming nearby to produce the regular spacing observed in hair follicles of the skin\textsuperscript{16}.

As the bud moves downward into the dermis, it splits into two structures, the outer root sheath (ORS) and the inner root sheath (IRS). The ORS maintains contact with the basement membrane, while the IRS becomes a channel for the developing hair (Figure 2). The first traces of hair shaft cells are visible in the central core around E18.5 in mice. Seven to eight days later a companion layer develops between the ORS and IRS, and each root sheath splits into three concentric cell layers. In the final stages of hair maturation, the protruding hair grows in these seven concentric rings\textsuperscript{16}.

Other Structures of the Skin: Teeth, Mammary Glands, and Nail

Tooth development is similar to that of hair, however the initial signals occur in the inverse direction. For hair, placode development is induced by the mesenchyme, while dental placodes result from an inductive signal from the epithelium\textsuperscript{1}. At E11 in the mouse, dental placode formation occurs in the dental lamina, a horseshoe shaped thickening of the epithelial tissue that demarcates the future dental arc (Figure 2)\textsuperscript{24}. It is believed that the four types of
mammalian teeth (premolar, molar, incisor, and canine) come from different initial placodes. The
dental placode invaginates into the mesenchyme to form a bud between E12 and E13, similar to
the process seen in hair bud formation\textsuperscript{18}. The enamel knot is then formed and is a distinct
thickening of epithelial cells as a result of the bud folding over itself and ceasing to proliferate\textsuperscript{37}. The cap is formed at E14-15 and is characterized by the epithelium adjacent to the knot growing
deeper into the mesenchyme. The mesenchyme is condensed and surrounded by the epithelium
forming cervical loops\textsuperscript{37}. The bell stage begins at E16 as the epithelium assumes its characteristic
tooth crown shape. In the late bell stage, the dental papillae cells differentiate to form
odontoblasts and the inner epithelial cells differentiate to form ameloblasts. The odontoblasts
give rise to dentin and ameloblasts give rise to the enamel matrix of the tooth. The dentin and
enamel found in teeth is unique compared to the keratinized epithelial structures formed in the
skin\textsuperscript{38}.

Mammary gland development begins at approximately the same time as teeth,
approximately E10.5. Two mammary lines, which can be likened to the dental lamina, appear
between the fore and hind limbs\textsuperscript{25, 26}. Within the next day (between E10.5 and E11.5), five pairs
of mammary placodes emerge in the anteroposterior axis\textsuperscript{25}. Around E12.5, the placodes begin
their invagination into the mesenchyme to become buds. These buds are considered specialized
dense mammary mesenchyme and express AR\textsuperscript{26}. The secondary mesenchyme underlies the
mammary mesenchyme and begins to form at E14 giving rise to the fat pad of the mammary
gland (Figure 2)\textsuperscript{1}. 
Nails also develop in a similar fashion. The first structure to develop is a nail field which develops into a placode from the thickened epithelium. Next, a transverse groove appears proximal to the placode. This groove develops into a deep fold, or invagination. At the base of this fold lie the dividing germinal matrix cells. These cells eventually produce a layer of keratinized cells that slide distally over the nail bed.

Figure 1-2. Epithelial-Mesenchyme interactions in the early development of various skin appendages. Skin appendages mostly follow a typical pattern of development from placode to bud. However, following bud formation specific signals drive differentiation of the various organs to develop into penile spines, hair, teeth, or mammary glands. The pictures of the penile spines are of a 20 day and 60 day FVB mouse expressing lacZ (blue) driven by the mouse AR enhancer[7]. Figure adapted from [18]
Overview of Current Study

Of these skin appendages, the development of the penile spines is the least studied. Two reports stated that spine development began around postnatal age 10 (P10)\(^\text{12,29}\); however there was no further detail given in regards to their development. The purpose of this study is to further clarify the early development of penile spines in mice and compare it to the better-characterized processes in other skin appendages. In particular, this study will address the histomorphological similarities and differences of penile spine development, formation, and maintenance compared to similar processes in hair and claws of the mouse. As discussed above, many of the other skin appendages, such as hair, teeth, mammary glands, and nail share similar characteristics in their early development. This is especially seen in their epithelial-mesenchyme interactions that cause the transition from placode to bud. It is reasonable then to assume that penile spines will share many of these same characteristics such as the epithelium thickening into a placode, followed by further modification into a bud, and translocation towards the mesoderm.

For direct histological comparison of penile spines to nails and hair, specimens were collected from wildtype FVB/NJ mice. The mice were euthanized at multiple post-natal time points. They were then dissected for their penises, skin on their back (hair), and paws (nails). Each specimen was paraffin embedded, sectioned, and stained using Milligan's trichrome Stain for muscle, collagen, and keratin. Milligan’s trichrome has not been used before to stain penile spines and another purpose of this study is to assess whether it can be a valuable method for staining these keratinized structures. Nail was chosen because after birth, it represents a largely fully developed keratinized structure. Thus the nail will serve as a control to validate distinctive keratin staining patterns observed in penile spines. Hair was chosen because some new hairs will go through their complete developmental process in a post-natal mouse. Hair development can also be characterized with the Milligan's stain and then compared to the development of penile
spines under the same stain. The stained specimens were then observed by light microscopy and pictures were obtained using Leica software.
Chapter 2
Materials and Methods

Tissue Preparation

An age series consisting of only males was bred from wildtype matings of FVB/NJ mice. The mice were fed solid food and water *ad libitum* and exposed to a 12 hour day/night cycle. The ages of mice collected were P2 (Day 1 of birth is equivalent to P0) P5, P7, P10, P12, P15, P20, P25, P30, P35, P40, and collection followed protocols approved by the PSU IACUC. Penises, back skin, and forepaws were removed and immediately fixed in 4% buffered paraformaldehyde for 24 hours. Paws were then decalcified for 1-2 weeks in 5% EDTA. All specimens were dehydrated in graded ethanol and chloroform baths prior to embedding into paraffin blocks. The specimens were embedded in such a way to ensure they were sectioned parallel to the sagital plane. An American Optical #820 microtome was used to section the blocks at 6 μm. The sections were mounted on Superfrost+ slides (VWR Scientific).

Histology

Sections were stained using the Milligan's trichrome stain allowing for a clear contrast between muscle, collagen, and keratin. The sections were first fixed with a 2.2% potassium dichromate (Sigma-Aldrich), hydrochloric acid, and 95% ethanol solution. Acid Fuchsin (Sigma) was applied to clearly contrast muscle from collagen. Phosphomolybdic acid (Sigma-Aldrich) was then applied which selectively extracts Acid Fuchsin from collagen but not muscle or
nuclei, followed by a solution of Orange G (Sigma), which is used to stain either erythrocytes or keratin. The sections were washed in 1% acetic acid, followed by staining with Fast Green FCF (Sigma) which stains collagen, and a final wash in 1% acetic acid. The sections were dehydrated in graded ethanol and chloroform washes and cover-slipped using Permount mounting media.

Stained sections were imaged using an Olympus BX50 light microscope attached to a Leica DFC450 digital image capture system. Images were assessed using Leica LAS V4.0 imaging software.
Chapter 3

Results

Nail Development

In order to test the efficacy of these protocols for visualizing keratinized structures in penile spines, nails were first stained using Milligan's trichrome. Figure 3-1a is a nail at P10. The dark pink, purple oval structure that overlays the epidermis, is the nail plate. Even at this age the nail plate completely covers the nail bed. There also is a distinctive yellow band in middle of the nail bed. Figure 3-1b is a nail from P35. The appearance is largely consistent in terms of relative length and development of the nail plate at this age compared to the P10 nail. However, the nail plate is thicker at this age, with more of the yellow color seen throughout. It can also clearly be seen that the keratinized cells in the nail plate are strung together in long sheets. These results seen in fully developed keratinized nails demonstrate that Milligan's trichrome is an effective stain for keratin.
Since hair develops in several waves, new hairs can be found at various developmental stages in the post-natal mouse. These developing hairs were recorded in order to compare their development to the keratinized penile spines. Figure 3-2a is a skin specimen from a P2 mouse. In this field of view the whole integument system can be characterized. Most superficial is the layer of keratinized stratified squamous epithelium. The keratin is represented by a dark purple color. Beneath that layer is the highly cellular dermis, containing many hair bulbs. The several bulbs that are most superficial have a dark purple protrusion. Since it is stained the same color as the epithelial layer, this protrusion represents the keratinized hair shaft.

The hair follicles develop further at P5. They grow longer towards the epidermis (Figure 3-2c). The roots of the hair remain the same dark purple color as they develop. By P40 the root continues to differentiate. Distinctions can be made between the IRS and the ORS (Figure 3-2d). In the middle of the IRS a yellow color begins to emerge. This same yellow color was also apparent in the middle of developed keratinized nail (Figure 3-1). These results show that
Milligan’s trichrome initially stains keratin a dark purple color. In fully developed keratinized structures, it stains the exterior of the hair shaft dark purple in the exterior while the interior of the structure is mixed with yellow staining.

Figure 3-2. Milligan’s trichrome stain of hair from the back skin of mice. Each picture represents a sagittal section through the skin. 

a. P2 skin is depicted. Hair roots are beginning to form and stained dark pink/purple. Epidermis (Ep) and dermis (D) are clearly visible.

b. A higher magnification of P2 skin. The hair follicles with their roots beginning to protrude is highlighted by the arrows.

c. P5 skin specimen. Hair roots are more numerous and longer.

d. P40 skin section. Root is longer and yellow color is visible in the center of the root where the arrow is pointing. Scale bar represents 50 µm.
Penile Spine Development

Penile spines initiate development later and prolong it relative to the hair and nails. In the P2 penis (Figure 3-3a) there is no indication of a developing placode. The epithelium is dark purple stained appearing as stratified squamous epithelium similar to the epithelium in the back skin (Figure 3-2a). There are, however, multiple round cells observed in the epithelium that were not seen in skin. These cells seem to stain clear with a darker staining central nucleus. At P5 (Figure 3-3b) these large clear staining cells are more numerous and have converged upon one another. Placodes are not yet identifiable at this age.

Placodes are visible at P7. These results are in agreement with Murakami (1987) who found that penile spines begin developing at one week after birth\textsuperscript{12}. In this figure, the epidermis stained a light pink color likely due to the keratinocytes located in the epidermis of skin. The dermis lies underneath this pink layer and is characterized by darker staining nuclei. The dermal cells appear to protrude into the level of the invaginating epidermis. By P10 (Figure 3-3d), the placodes grow larger with more dermal cells occupying its space. In this figure the epidermis did not stain as clearly as in other figures, however the placodes filled with dermal cells are still clearly visible.
Figure 3-3. Early development of penile spines. Specimens are sectioned in the sagittal plane. a. P2 penis section. No development of penile spines is detectable. b. P5 penis section illustrating the continued lack of spine development at this stage. c. Placodes present in P7 penis (arrows). Dermal cells invade the invaginated epithelium. Epidermis (Ep) and dermis (D) are clearly distinct from each other. d. P10 penis section shows increased development of placodes indicated by the greater number of dermal cells. Scale bar represents 50µm.
The P12 penis (Figure 3-4a) displays larger placodes with even more dermal cells. The invaginated epidermis starts to engulf the invading placode. By P15 (Figure 3-4b) the epidermis has fully enclosed the dermal cells, forming the bud or dermal papillae.

After the bud stage, the penile spines initiate their later stages of development. At P20 (Figure 3-4c), dark purple structures begin to protrude from the center of the bud. This same color was observed in both nail (Figure 3-1) and hair (Figure 3-2) and represents the keratinized portion of each structure. In Figure 3-4d, the spines begin to grow larger and extend towards the epidermis. They closely resemble the roots of hair seen in early development (Figure 3-2b).

At P43 (Figure 3-5c), the penile spines are close to reaching their final maturity. The spine on the far right is starting to show a lighter yellow color at its tip. This color is similar to the yellow stain in fully developed keratinized nail (Figure 3-1). In Figure 3-5d, the spines are now protruding out of the epidermis. The yellow color is in the interior of the spine and is enclosed by a dark pink color. This represents a fully mature penile spine. The cells of these spines also seem to be oriented in long sheets, similar to those seen in nails (Figure 3-1b).
Figure 3-4. Middle stage development of penile spines. Specimens are sectioned in the sagittal plane. a. P12 penis specimen with increased growth of placode. Arrow indicates the epidermis beginning to enclose the dermal cells. b. P15 penis specimen. Buds have now formed (arrows) as the epidermis has fully enclosed the dermal cells. c. P20 penis specimen shows the first sign of keratinized structure. These stain dark pink at each arrow. d. P25 penis specimen. The spines continue to increase in size. Scale bar represents 50 µm.
Figure 3. Late stage development of penile spines. Specimens are sectioned in the sagittal plane. 

a. P35 penis specimen. Prepuce (PP) begins separate from epidermis. 
b. P35 penis specimen. Note that the PP is fully separated from the glans. Spines are close to reaching their full length. 
c. P43 specimen. Arrow points to the first sign of yellow color seen in the spine. 
d. P63 penile spine. Fully developed stage, arrow points to a spine with yellow in its interior and dark pink in its exterior. Scale bar represents 50 µm.
Chapter 4

Discussion

Milligan’s Trichrome Stain for Keratin

One of the main purposes of this study was to evaluate the efficacy of using Milligan’s trichrome stain to document keratin expression and formation in skin appendages. The results from this study have shown that it is a useful stain for this purpose. Hair and nails are known to express keratin in both the hair root and the nail plate\textsuperscript{32-36}. The hair root (Figure 3-2) and the nail plate (Figure 3-1) all stained a very distinctive dark purple color. Keratin is also expressed in the epidermis of skin. This stain made it easy to distinguish the hair, nail, and penile spines from the keratinized epithelium.

In the fully developed hair and nail, a yellow color also appeared in the center of the keratinized structure (Figure 3-2d and Figure 3-1). In hair this could potentially be the IRS that is stained yellow. Since this is the developing hair shaft, the cells are rapidly dividing stem cells\textsuperscript{32} so they may stain differently or have different types of keratin proteins compared to the non-dividing cells in the ORS. Keratins can be divided into two categories, soft and hard keratin. Soft keratin, also known as epithelium-like keratin, is solely expressed in the epithelium of skin, while hard keratin, or hair-like keratin, is the only type of keratin expressed in the exposed portion of hair\textsuperscript{35,36}. However the proximal portion of the root near the hair follicle expresses both keratins\textsuperscript{36}. Similarly, the nail plate also expresses both hard and soft keratins\textsuperscript{36}. However, the spatial distribution of these keratins can be highly variable, with some regions expressing only soft, only hard, or co-expression of both keratins\textsuperscript{35}. In the P10 nail (Figure 3-1a) it appears that the distal
portion of the nail plate is only stained dark pink while the interior of the proximal end of the plate appears to only stain yellow. In the P35 nail (Figure 3-1b), it appears the dark pink and yellow colors are interspersed throughout the length of the nail plate. This pattern could suggest that Milligan’s trichrome stain distinguishes between hard and soft keratins by staining them either yellow or dark pink/purple.

Keratin is a specific type of protein from the intermediate filament class. There are five classes of intermediate filaments; including type I, which are acidic keratins and type II, which are basic keratins. Hard keratins and soft keratins express both of these types. Hard keratins can be further broken down to include type Ia and IIa proteins and are classified as having a higher cysteine content from soft keratins. About 7.6% of the amino acid sequence is cysteine, which allows for an increased amount of disulfide bridges in the folded keratin protein, giving it a harder and more durable structure. Soft keratins are broken down between type Ib and IIb and their amino acid structure is classified by being composed of 2.9% cysteine.

Based on the results presented here (Figures 3-3, 3-4, and 3-5), it is clear that the Milligan’s trichrome stains epithelium keratinocytes light pink/purple. This may indicate that the soft keratin stains pink/purple and the hard keratin stains yellow. This would accord with the change in staining observed between the P10 nail, in which only a small amount of yellow color is visible, and the P35 nail where yellow staining is ubiquitous in the nail plate. As the nail ages it is expected that a greater proportion of hard keratin relative to soft keratin would be expressed.

**Penile Spine Development**

One of the main findings from the penile spine results is that placodes first developed seven days after birth (Figure 3-3c), and buds developed around 15 days after birth (Figure 3-4b).
Placode growth was determined by an invagination of epidermal cells that create a ‘pocket’ into which dermal cells invade. Buds were defined as fully enclosed dermal cell pockets in the epithelium. These interactions between the epithelium and dermal layers are very characteristic of other skin appendages, such as hair, nail, teeth, and mammary glands\textsuperscript{18}. In these appendages, the thickened epithelium that forms a placode invaginates down into the mesenchyme, as observed here during the formation of penile spine placodes.

Using the findings of keratin colored staining from hair and nail, it was observed that keratinized spines first begin to protrude from the epithelium at P20 (Figure 3-4c). The same dark pink/purple that is hypothesized to be soft keratin in hair and nail first appears in the penile spines at this age. The spines continue to grow towards the epithelium in a manner similar to the way that the developing hair root does up through P35 (Figure 3-2, 3-4c, 3-5b). At P43 (Figure 3-5c), the spines begin the process of their final maturation as the yellow stain begins to appear in the middle of the spine. The yellow stain, which may indicate hard keratin, was not observed until hair follicles reached maturation (Figure 3-2d). The penile spines reached their full maturity at P63 (Figure 3-5d). At this age, the region of yellow staining expands and occupies the whole interior of the spine, similar to the IRS of the mature hair root and the nail plate.

**Limitations and Future Directions**

One of the limitations of this study was its use of only post-natal specimens. For the study of developing penile spines, post-natal specimens are sufficient because the entire course of their development occurs after birth. Post-natal specimens were also sufficient for studying the use of Milligan’s trichrome stain on fully developed keratinized structures like hair and nails. Despite the fact that the initiation of hair development occurs \textit{in utero}, comparisons could still be
made between hair and penile spine development, because hair formation is a continuous process occurring in several waves of growth. However, even in newly developing hair roots in post-natal mice, a hair follicle has already been formed. Further work is required to compare the early stages of penile spine formation to similar stages of hair and nail development in embryonic mice.

Placodes are known to start developing in hair at E13.5 and progress quickly to buds by E18. Observations of specimens at these ages will provide a more appropriate and definitive comparison with post-natal penile spine development. While hair and nails are two of the more prominent keratinized skin appendages in mammals, however various keratinized appendages also occur in other amniotes. Bird feathers and reptile scales can also serve as keratinized structures to compare to penile spine development. A more comprehensive study is still required to compare penile spine development to the diverse array of skin appendages to see if they share the same epithelial-mesenchymal (dermal) interactions and patterns of gene expression.

Aside from suggestions for changes to the current study, these results also raise several new questions and future directions. Since it was determined that histomorphologically, penile spine development was largely similar to other skin appendages from the placode formation to bud transition, this raises the question as to whether similar genetic signals are involved. Beyond surveying similarities and differences in genetic signaling, it is also possible to conduct experiments to determine the capacity of different tissues to induce or respond to signals that spur penile spine formation. As discussed above, hair development begins by signals from the mesenchyme to the epithelium beginning epithelial invagination to form a placode. However this is not the case in tooth development, which requires an inductive signal from the epithelium to the mesenchymal tissue. It appears that the dermal cells extend superficially to invade the epithelial layer in penile spines at P7 and P10 (Figure 3-3a-b); however this is not a sufficient indication of the source or targets of the signaling tissues. A better experiment would involve tissue recombination in which the epidermis of the glans penis is replaced by a regular skin
epidermis. If the inductive signals come from the dermis, then penile spines are still capable of being produced with the alien epidermis. Tissue recombination experiments have already shown this for developing placodes in mouse hair, chicken feathers, and lizard scales.\textsuperscript{33}

Aside from the direction of inductive signals, future experiments can also test what the actual signals are that induce penile spines. FGF and BMP inhibitors are essential in hair development for the formation of placodes.\textsuperscript{21} Wnt and Shh are also essential in developing the follicle after placode formation.\textsuperscript{23} Either immunohistochemistry (IHC) or \textit{in situ} hybridization could be used to determine the identity, location, and timing of proteins and genes expressed during penile spine development. IHC experiments were attempted for this specific purpose. Antibodies for Shh, Wnt, ectodysplasin-A, ectodysplasin-A receptor, AR, and proliferating cell nuclear antigen were tested in order to determine their efficacy for determining protein expression in developing penile spines. The preliminary results proved inconclusive and were thus not included in this study's results. However, further work is necessary to refine protocols to enable detection of protein and gene expression during penile spine formation. The proteins previously studied should be explored again using IHC because of the histological similarities in development of penile spines seen in this study to other skin appendages.

The results that indicate Milligan's trichrome may stain differentially for hard and soft keratins indicates the need for further future experiments. To test whether or not the yellow stain is hard keratin and the dark pink/purple stain is soft keratin, several experimental methodologies can be pursued. The parts of nail that stain only yellow in P10 nail and the parts that stain only dark pink in P10 nail could be dissected from the paw and their amino acid content could be analyzed using mass spectrometry. This technique was used to first distinguish differences between hard and soft keratins.\textsuperscript{41} Lynch et al. also developed several mouse antibodies that differentially recognize hard and soft keratins. Using antibodies of this nature, IHC experiments
could be conducted in hair, nails, and penile spines to determine the relative concentrations of hard and soft keratin in each structure\textsuperscript{40}.

**Conclusion**

The main findings of this study are that Milligan’s trichrome stain can be used to effectively stain for keratinized structures and distinguish them from muscle and collagen in hair, nails, and penile spines. Milligan’s trichrome stain may also differentially stain hard and soft keratins and could potentially be used to track the changes in their relative expression during spine maturation. Penile spines development is histomorphologically similar to other skin appendages. Placodes appear at P7, buds appear by P15, and the keratinized spines appear at P20. By P63 they are fully formed keratinized spines that are exposed to the exterior.
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