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Periodic patterns in Rodentia: Development and evolution

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Abstract

Mammalian periodic pigment patterns, such as spots and stripes, have long interested mathematicians and biologists because they arise from non-random developmental processes that are programmed to be spatially constrained, and can therefore be used as a model to understand how organized morphological structures develop. Despite such interest, the developmental and molecular processes underlying their formation remain poorly understood. Here, we argue that Arvicanthines, a clade of African rodents that naturally evolved a remarkable array of coat patterns, represent a tractable model system in which to dissect the mechanistic basis of pigment pattern formation. Indeed, we review recent insights into the process of stripe formation that were obtained using an Arvicanthine species, the African striped mouse (*Rhabdomys pumilio*), and discuss how these rodents can be used to probe deeply into our understanding of the factors that specify and implement positional information in the skin. By combining naturally evolved pigment pattern variation in rodents with classic and novel experimental approaches, we can substantially advance our understanding of the processes by which spatial patterns of cell differentiation are established during embryogenesis, a fundamental question in developmental biology.

Keywords

emerging models; evolutionary developmental biology; pigment patterns

1 | INTRODUCTION

Of the various traits showing spatial specific differences in the skin, mammalian pigment patterns are one of the most fascinating, due to their visual accessibility, diversity and obvious relevance from an adaptive perspective.[1,2] The general appearance of pigment patterns allows a simple classification[3] as either stochastic, such as the variegated and patchy nature of a calico cat,[4] or organized, as in regularly spaced stripes in a tabby cat,[5,6] or the unique facial appearances that characterize closely related species of African

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CONFLICT OF INTEREST

None.

monkeys.[7] Classification as stochastic vs. organized is also useful in conceptualizing differences in developmental origins. Most variegated patterns arise due to random events during embryonic development, such as X inactivation or the death of melanocyte precursors in a calico cat.[4] By contrast, organized patterns are a consequence of developmentally programmed processes by which adjacent groups of cells acquire differences in their behaviour and fate, in a way that is both repeatable and heritable. Among organized patterns, those showing periodicity (eg, zebra stripes or cheetah spots) have been of long-standing interest to mathematicians and biologists, because they can be used to understand the theoretical underpinnings and molecular processes by which repetitive organized morphological structures develop.[8,9] While studies in non-mammalian vertebrates (eg, zebrafish and chicken) have provided tremendous insights into these processes,[8-13] the mechanisms underlying periodic pattern formation in mammals remain largely unknown.

Previous studies have used Mendelian variation in pigment patterns to identify some of the genes and pathways involved in felid tabby pattern[6,14] and have also highlighted the challenges of studying pattern biology in systems outside of a controlled laboratory setting. Although felids such as the Panthera (lions, tigers, leopards and jaguars) are easily recognizable examples of unique pigment patterns in closely related species, access to samples and concerns regarding conservation, ethics, and cost limit the opportunities for experimental investigation. An alternative approach exemplified by some of our recent work is based on rodents.[15] The most taxonomically diverse order of mammals, rodents represent an attractive and tractable group in which classic and modern approaches can be combined to dissect the molecular and developmental mechanisms underlying mammalian pigment pattern formation.

In rodents, periodic patterns have evolved in members of at least 5 different families (Muridae, Sciuridae, Geomyidae, Dipodidae, Nesomyidae and Cricetidae). Within the subfamily Murinae (family Muridae), which comprises old world mice and rats, there is a mono-phyletic clade of closely related species, termed the Arvicanthines, whose members have evolved an extensive array of periodic patterns, which vary in number, position, and complexity (eg, spots/stripes vs. only stripes) (Figure 1).[15,16] We and others have demonstrated the feasibility of establishing breeding colonies with Arvicanthine species,[17,18] which opens the door for performing controlled experiments and the development of molecular tools. In addition, Arvicanthines are very closely related to laboratory mice,[17] the premier model species in mammalian/skin research, allowing for powerful comparative approaches and making many of the currently available tools transferable, as we discuss below.

2 | BACKGROUND: SKIN BIOLOGY

The skin is the largest organ of the body and has many important functions, from protecting the body from infection and desiccation to playing key roles in regulating temperature, secretion, and camouflage. During embryogenesis, the skin arises by interactions between ectodermally derived epithelial cells, which will constitute the epidermis, and underlying mesenchymal cells derived from the mesoderm, which eventually form the dermis. When mesenchymal cells are in contact with the overlying epithelial cells, a reciprocal signalling

crosstalk begins to unfold, leading to the formation of different cutaneous appendages such as hair/feather follicles and various types of glands.[19] Although this general developmental program is responsible for forming the integument that covers the entire embryo, it shows considerable variation among different body regions within individuals, which lead to localized changes in skin thickness, pigmentation, and types of cutaneous appendages.[20,21] For example, many vertebrates display regional colour variation, with dorsal hair being lighter than hair found in the ventral surface.[22-24] In mammals, mammary and eccrine glands tend to be restricted to the ventral surface of the body and to the palmoplantar skin of the feet, respectively, and in birds, there are regions devoid of feathers and marked differences in the scales of the dorsal and ventral surfaces of the feet.[24-26] A number of studies using mouse and chicken as models have elucidated some of the genes controlling these and other local spatial differences found in the skin, revealing key insights into the molecular mechanisms by which regionalization is established during embryogenesis (reviewed in ref 21).

3 | STRIPED RODENTS AS A MODEL FOR STUDYING MECHANISMS OF PERIODIC PATTERN FORMATION

In a recent study, we capitalized on several of the aspects mentioned above, and used a species of Arcivanthine rodent, the African striped mouse (*Rhabdomys pumilio*), to investigate the developmental mechanisms underlying the formation of the periodic stripes, a common pattern in mammals.[18] Striped mice have a coat characterized by the presence of four dark and two light dorsal stripes arranged in a dark-light-dark pattern (Figure 2A). To understand how and when these differences arise, we analysed striped mice samples from different stages and found that the stripe pattern is established during late embryogenesis (Figure 2B). Although we found that pigment-producing cells (ie, melanocytes) were present in both light and dark stripes and at similar numbers, as revealed by immunohistochemistry for KIT,[18] those found in light stripes fail to differentiate, as determined by immunohistochemistry for Mitf (Figure 2C,D). As a result, they produce little or no pigment. Using RNA-sequencing, we compared the abundance of transcripts found during the formation of light and dark stripes and found that the transcription factor *Alx3* was expressed at much higher levels in skin of the light than in the dark stripe. Analysis of *Alx3* expression during skin formation, prior to the appearance of stripes, revealed that this gene was already expressed in the region of the dorsal skin where stripes will subsequently form, suggesting that this gene may be influencing the development of melanocytes and the production of pigment.

To test the potential role of *Alx3* experimentally, we used ultrasound-guided injections to deliver a lentivirus carrying a copy of *Alx3* to developing mouse embryos in utero: using this technique, we were able to inject the lentivirus into the amniotic cavity of early embryos, prior to neural tube closure, allowing for specific infection of both epidermis and cells derived from the neural crest (eg, melanocyte progenitors) (Figure 2E,F). A few days later, when we analysed the samples, we found that, indeed, those melanocytes infected with the *Alx3*-carrying virus did not differentiate (Figure 2G,H), recapitulating the process occurring during the formation of the light stripe.[18]

To obtain a more detailed understanding of the mechanism by which *Alx3* is altering melanocyte development, we used a combination of bioinformatic approaches and protein-binding assays to show that *Alx3* directly represses one of the key regulators of melanocyte differentiation and pigment production—a transcription factor called *Mitf*— by binding directly to its promoter. Indeed, the *MITF* promoter contains an abundance of putative *Alx3* binding sites, several of which are conserved across mammals.[18]

To directly explore whether the mechanism in striped mice might exist in other rodents, we analysed skin biopsies from chipmunks, which last shared a common ancestor with African striped mice about 70 mya and independently evolved a similar dorsal stripe pattern. We found that *Alx3* is also found at high levels in chipmunk skin containing light hair, indicating that regulated expression of *Alx3* is a patterning mechanism that has repeatedly evolved for the same purpose in different rodent groups.

4 | ACQUIRING POSITIONAL INFORMATION IN THE SKIN

As was previously suggested by Kaelin and Barsh,[3,6] it is useful to conceptualize *specification* and *implementation* as key steps in the development and acquisition of pigment patterns. In this context, *specification* refers to the developmental mechanisms in embryogenesis that establish the positional boundaries of the future pattern, while *implementation* refers to the mechanisms by which groups of previously specified cells give rise to visible differences in pattern. Distinguishing between specification and implementation is useful because different pigment patterns may arise from changes in the developmental mechanisms during specification, but may still utilize common mechanisms of implementation. For example, domestic cats,[6] cheetahs,[6] and striped mice[18] all exhibit increased levels of the gene *Edn3* in regions that contain dark hair, relative to other regions of the skin, yet their pigment patterns are very different.

4.1 | Implementing periodic patterns

Our work on *Alx3* in striped mice exemplifies a melanocyte-autonomous mechanism of implementation, since increased expression of *Alx3* within a melanocyte causes downregulation of *Mitf* within the same cell. But implementation also involves mechanisms that are melanocyte non-autonomous: our RNAseq experiments provided evidence that the well-known paracrine signalling proteins Agouti and Endothelin 3 also participate in modulating hair colour. Thus, implementation of pigment pattern in striped mice involves an interplay between melanocyte-autonomous and non-autonomous pathways. An important question for the future is to determine whether the pattern specification mechanisms that lead to differential expression of *Alx3*, *Agouti* and *Edn3* proceed by a common or by independent and parallel pathways.

To learn more about pigment pattern specification, it is helpful to consider potential mechanisms in the context of gene regulation. Establishment of the spatially and temporally restricted gene expression networks that implement stripe patterns is likely mediated by the interaction between different transcription factors and DNA-binding sites within *cis*-regulatory modules of promoters and enhancers. For example, a transcription factor that might activate the expression of *Alx3* in light stripe melanocytes must act by binding to a

cis-regulatory motif; as a corollary, this transcription factor is either not expressed in dark stripe melanocytes or cannot access the same binding motif. These considerations suggest a potential experimental genomic approach, based on techniques such as ATAC-seq,[27] FAIREseq,[28] and DNAse-seq,[29] that allow genomewide profiling of chromatin landscapes of specific tissues or different cell types within a tissue. Due to the close phylogenetic proximity between laboratory and striped mice, many antibodies directed towards the former species can be successfully used in the latter, making it possible to incorporate conventional approaches (eg, FACS) to isolate the cell types that are likely relevant for the establishment of this phenotype (ie, dermal papilla cells and melanocytes). Thus, it seems possible that obtaining genomewide chromatin profiles from the different stripe tissues and/or cells during growth will reveal *cis*-regulatory modules that connect pattern specification to pattern implementation.

Candidate *cis*-regulatory modules identified using the methods mentioned above have to be coupled with approaches aimed at testing their functional significance, which range from in vitro massively parallel reporter assays to smaller scale in vivo reporter assays.[30,31] Although we anticipate the development of CRISPR/Cas9-mediated genome editing tools in striped mice (see below), the laboratory mouse can be leveraged as a powerful tool to dissect enhancer function, either by performing reporter assays in one of the many murine cell lines already available or directly in vivo. Together, the profiling of chromatin landscapes in different stripes coupled to the functional characterization of the differences seen, will provide insights into the regulatory events controlling the differential expression of pigment pattern implementation factors.

4.2 | Specifying periodic patterns

As highlighted above, our initial RNAseq screen indicated that the *Alx3*, *Asip* and *Edn3* participate in implementing the striped pattern in striped mice. However, the positional events that establish the pattern itself early in development and the molecular identity of the stripe-inducing signal remain unknown. Do all rodents have a stripe-inducing signal but only striped rodents have the ability to respond to such a signal? Alternatively, did striped rodents evolve a unique stripe-inducing signal that other rodents don't have? Below, we discuss how these questions can be answered through a combination of classic transplant experiments and cutting-edge genomic approaches.

Classic transplantation experiments aimed at uncovering the mechanism of action of the *Agouti* gene, which controls the switch from the production of black (eumelanin) to yellow (pheomelanin) pigment, have shown that dorsal and ventral embryonic skin from a^t/a^t mice, a strain that has a dark dorsum and a light ventrum, have the ability of producing black and yellow hair, respectively.[32] In addition, epidermal-dermal recombination experiments showed that this effect is mediated by the embryonic dermis.[33] Candille et al. performed similar experiments and found that the information required for establishing this positional identity is present as early as embryonic day 12.5.[34] In striped mice, variations of these experiments can be performed, making it possible to dissect the temporal and spatial origin of the stripe-inducing signal. For example, grafting dorsal skin from multiple embryonic stages (E10-E16) into nude BALB/c mice, and establishing the earliest stage in which dorsal

tissue is capable of generating a striped pattern, may provide insights into when in development this signal operates. In addition, epidermal-dermal recombination experiments between dorsal (future stripes) and ventral (non-striped) skin will likely reveal whether the epidermis or the dermis conveys the positional information to induce stripe formation. Thus, transplant experiments may provide valuable insights into the *temporal* and *spatial* mechanisms operating during stripe pattern formation.

Once the information on the temporal and spatial origin of the stripe-inducing signal is obtained, it will be possible to probe the relevant stages and tissues more deeply, using approaches aimed at assessing differences in transcriptional activity (eg, single-cell RNA-sequencing), in order to understand the molecular basis by which the stripe patterning mechanisms are established. In this sense, like with other ideas discussed here, the parallel generation of single cell transcriptional profiles from *Mus* skin at comparable developmental stages, coupled to existing databases,[35] will make it possible to obtain a transcriptional landscape of the different cell types present in non-striped embryonic skin. By comparing single cell transcriptional profiles of non-striped and striped embryonic skin, it may be possible to tease apart the transcriptional mechanisms that distinguish different cell types, as defined by well-established molecular markers, from those that establish the stripes.

5 | FUNCTIONAL APPROACHES TO STUDYING PIGMENT PATTERNS IN RODENTS

As outlined above, the powerful genetic and molecular tools available for the laboratory mouse makes this species ideal for testing enhancer function using reporter assays and performing gain-of-function experiments aimed at altering coat colouration, ideally in a spatially specific fashion. However, the feasibility of establishing and maintaining breeding colonies of striped mice in pathogen-free animal facilities opens the door for developing approaches aimed at interrogating gene and enhancer function directly in this species. For example, we and others have successfully used ultrasound-guided in utero injections to transduce lentiviruses into different populations of developing skin cells from various species, including laboratory mice and deer mice.[18,22] By achieving efficient selective transduction of epidermal and dermal cells, in which genetic alterations are stably incorporated and propagated, this approach allows for rapid assessment of gene function and can be used in a wide variety of taxa, including striped mice.

The advent of CRISPR/Cas9 technology has opened the door for performing a wide variety of genome editing experiments in both “traditional” and “non-traditional” model systems, allowing a wide variety of modifications, including gene and enhancer knockouts, transcriptional activation and repression, and insertions of large genomic regions. Furthermore, recent studies in laboratory mice have achieved high genome editing efficiencies by delivering the CRISPR/Cas9 cocktail directly into zygotes inside the female oviduct, using electroporation[36] or adeno-associated viruses,[37] thereby bypassing some of the hurdles associated with the traditional approach, such as the use of sophisticated micromanipulation equipment for ex vivo zygote microinjection and embryo transfer, usually performed by highly trained personnel. Incorporating these approaches in striped

mice holds the promise of achieving precise and efficient genome modification to probe developmental mechanisms underlying pigment pattern formation.

6 | CONCLUSION

The mammalian skin displays a remarkable variation in structure and function within individuals and across species. Due to its clinical relevance, the molecular mechanisms underlying its formation, maintenance, and renewal have been studied for decades and are therefore well characterized, constituting a solid framework for studying how these processes are locally regulated and how these processes vary in different species. By capitalizing on naturally evolved pigment pattern variation in rodents, a group that is amenable to captive breeding and experimental manipulations, it is now possible to combine cutting edge and classic approaches to dissect the detailed molecular and developmental mechanisms by which positional information is acquired and modified in tissues, two long-standing questions in developmental and evolutionary biology. Focusing on genomic and molecular mechanisms underlying rodent/mammal ectoderm development and evolution holds the promise of uncovering fundamental biological aspects with the potential to impact various fields, from evolutionary genomics and developmental biology to clinical sciences.

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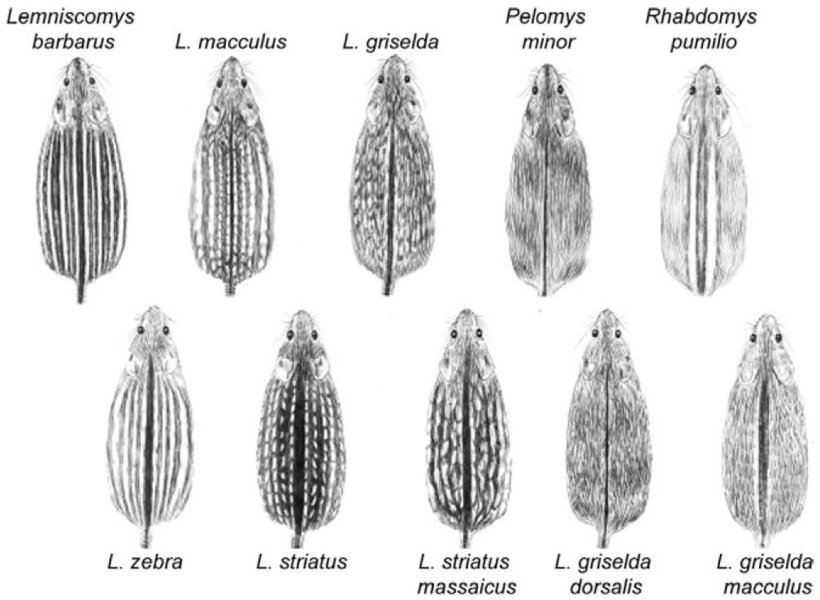

FIGURE 1.

Representatives of the Arvicanthine group, illustrating the diversity of coat patterns found in this group. Drawings modified from Ref [16]

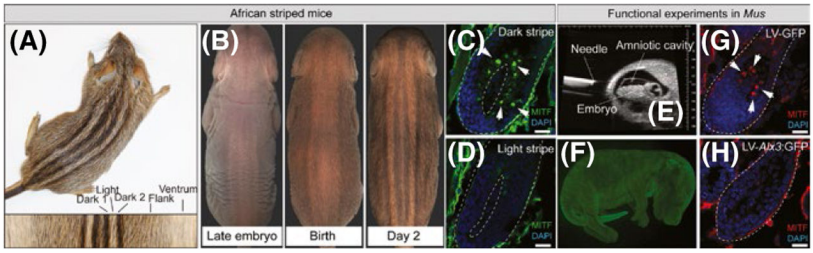

FIGURE 2.

(A) African striped mice (*Rhabdomys pumilio*) have dorsal light and dark stripes. (B) The stripe pattern is established during embryogenesis. (C-D) Hair follicles in dark stripes differ in the number of differentiated melanocytes, as seen by the marker MITF. (D-E) Ultrasound-assisted injections in *Mus* embryos (D) generates robust transduction of developing skin cells (E). F-G. A lentivirus carrying *Alx3* decreases the number of differentiated melanocytes, as seen by MITF stains. Scale (C-D; G-H) 100 μm