Not Black and White: BMP Signaling Drives Melanocyte Differentiation Down Stream of Stem Cell Activation

Nicole Rai Infarinato
NOT BLACK AND WHITE: BMP SIGNALING DRIVES MELANOCYTE DIFFERENTIATION DOWNSTREAM OF STEM CELL ACTIVATION

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NOT BLACK AND WHITE: BMP SIGNALING DRIVES MELANOCYTE DIFFERENTIATION DOWNSTREAM OF STEM CELL ACTIVATION

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Tissue stem cells (SCs) maintain, regenerate, and repair the body over the course of an organism’s lifetime. To preserve their long-term function, SCs must exert precise control over their cell state dynamics as they move from quiescence to activation and commit to full differentiation. My graduate research has been centered on investigating the molecular mechanisms that fuel these transitions in melanocyte stem cells (McSCs), a unique neural crest-derived SC population located in the hair follicle (HF). Through periodic bouts synchronous with HF cycling, quiescent McSCs become activated to proliferate, giving rise to committed proliferative progeny (McCP) that differentiate into mature pigment-producing melanocytes. The signaling factors and gene expression programs that orchestrate these cellular changes are still incompletely understood. To elucidate new insights into this process, I developed fluorescence-activated cell sorting strategies to isolate quiescent, activated, and differentiating McSC lineage cells from the mouse skin at discrete stages of the hair cycle. I then performed single cell RNA-sequencing (scRNA-seq) to reveal the evolving transcriptional signatures of the lineage with high resolution. Comparative bioinformatic analyses suggested that BMP and WNT signaling increase concomitantly throughout differentiation. I then sought to understand the role of BMP signaling in McSC lineage progression and whether this pathway engages in crosstalk with WNT signaling. To do so, I performed conditional lineage-specific genetic ablation of Bmpr1a to extinguish BMP signaling, which resulted in gray hair. However, McSCs remained intact and functional in these mutant animals, indicating dysfunction in their differentiating progeny. ScRNA-seq and pseudotime analysis of Bmpr1a null cells indicated a block in the differentiation program just downstream of the early McCP stage, and I detected further signs of melanocyte immaturity using immunofluorescence and electron microscopy analyses. Seeking mechanistic understanding, I interrogated changes in transcription factor expression at this blockage point. I found reduced nuclear levels of the master regulator MITF and WNT mediator LEF1. Using WNT mutant mouse models, cell culture systems, and chromatin profiling, I provide evidence demonstrating cooperation of BMP and WNT signaling to trigger complete differentiation of McCP into melanocytes through MITF and LEF1 activity. Altogether, I have generated a thorough characterization of the transcriptional and chromatin landscape changes that temporally define McSC lineage commitment in vivo. My findings underscore a critical role for signaling through BMPR1a to achieve full melanocyte differentiation in the HF. These findings raise intriguing questions about the role of BMP in hair and coat color variation, age-related hair graying, and melanoma initiation and progression.
For Ryan, who shows me what courage and ambition looks like.

Thank you for helping me do hard things.
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Id1/2/3  Inhibitor of DNA Binding
IMF  Immunofluorescence
IP  Intraperitoneal (injection)
k-NN  k-nearest neighbors
Kb  Kilobases
KO  Knockout
LacZ  β-galactosidase
Lef1/LEF1  Lymphoid Enhancer Binding Factor 1
Mclr/MC1R  Melanocortin 1 receptor
McCP  Melanocyte stem cell committed, proliferative progeny
McSC  Melanocyte stem cell
Mitf/MITF  Microphthalmia-associated transcription factor
Msx1/2  Msh homeobox
NF1  Neurofibromin 1
NRAS  Neuroblastoma RAS viral oncogene homolog
Oca2  Oculocutaneous albinism II
ORS  Outer root sheath
P  Postnatal day
p53  Tumor protein P53
Pax3/PAX3  Paired box 3
PBS  Phosphate buffered saline
PCA  Principle component analysis
PI3K  Phosphoinositol 3-kinase pathway
PMEL  Premelanosome protein
POMC  Proopiomelanocortin
pP38  Phosphorylated p38 MAP kinase
pSMAD1/5/9  Phosphorylated SMAD1/5/9
PTEN  Phosphatase-and-tensin homologue
qMcSC  Quiescent melanocyte stem cell
R26YFP  Rosa-26 yellow fluorescent protein
RAC1  Ras-related C3 botulinum toxin substrate 1
RBP-J  Recombination signal binding protein for immunoglobulin kappa J
RFP  Red fluorescent protein
RT-qPCR  Real time quantitative polymerase chain reaction
SC  Stem cell
SCF  Stem cell factor (also known as KIT ligand)
scRNA-seq  Single cell RNA-sequencing
SDF-1  Stromal cell-derived factor 1
SHH  Sonic hedgehog
Slc45a2  Solute carrier family 45 member 2
Sox10/SOX10  Sex determining region Y-box 10
SMAD  C. elegans Sma and the Drosophila Mothers against decapentaplegic
TACs  Transit-amplifying cells
Telo  Telogen
TCF  T cell factor
TGF-β  Transforming growth factor beta
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TPM</td>
<td>Transcripts per million</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription start site</td>
</tr>
<tr>
<td><em>Tyr</em>/TYR</td>
<td>Tyrosinase</td>
</tr>
<tr>
<td><em>TyrCreER</em></td>
<td>Tyrosinase estrogen receptor Cre recombinase</td>
</tr>
<tr>
<td>TYRP1</td>
<td>Tyrosinase-related protein 1</td>
</tr>
<tr>
<td>UMAP</td>
<td>Uniform manifold approximation and projection</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet (light/radiation)</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless-related integration site</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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CHAPTER 1:
INTRODUCTION
1.1 Melanocytes and their embryonic origins

Looking across the animal kingdom, there is astonishing variety in hair, fur, skin, and feather pigmentation and patterning. These shades and their unique organization have myriad functions including protection from ultraviolet (UV) light, thermoregulation, camouflage, sexual selection, and communication (Caro and Mallarino, 2020). Underlying this phenotypic diversity are melanocytes, a specialized cell type found in vertebrates that synthesize and transfer black-brown eumelanin and red-yellow pheomelanin pigment (Mort et al., 2015). Given the overt visible effects induced by changes in this lineage, melanocytes and their precursor cells have long been an attractive system for developmental and cell biologists and geneticists to harness. From the wide array of coat colors in “fancy mice” to animals recapitulating human cancer and disease, mice continue to be an excellent model organism in which to study this unique lineage.

The cells that terminally differentiate into melanocytes initially emerge during embryonic development from the trunk region of the neural crest. A small number of founder progenitor cells called melanoblasts are specified in a process governed chiefly by WNT signaling (Luciani et al., 2011; Thomas and Erickson, 2008). WNT induces neural crest formation and also directs downstream specification of melanoblasts. WNT1 and WNT3a ligands are expressed in the dorsal neural tube, and double knockout (KO) mice show defects in melanocyte specification, along with other neural crest-derived lineages (Ikeya et al., 1997). Consistently, neural crest-specific KO of downstream WNT mediator β-catenin using Wnt1-Cre causes loss of melanocytes (Hari et al., 2002; Hari et al., 2012). BMP signaling appears to act antagonistically, and BMP4 is downregulated in the neural tube at the time of melanoblast specification. Cultured neural crest cells treated with WNT3a adopt a melanocyte fate, while BMP4 drives cells instead towards a neural fate (Jin et al., 2001). Transcription factors (TFs) SOX10 and PAX3 are expressed in both
melanocyte and glial fated cells, while WNT/β-catenin activates the master regulator of the melanocyte lineage microphthalmia-associated transcription factor (MITF) (Dorsky et al., 2000; Takeda et al., 2000). Differentiation between these two lineages occurs via FOXD3, which represses MITF to favor neuron and glial cell fates (Thomas and Erickson, 2009). SOX10 and PAX3 then in turn regulate MITF (Potterf et al., 2000).

Around embryonic day (E) 9 in mice, melanoblasts begin to depart from the neural crest and embark on a spectacular migratory journey to colonize their destination niches throughout the body. They move along two major routes: the dorsolateral and ventral pathways. In the more well-characterized dorsolateral pathway, melanoblasts travel between the somites and ectoderm in what appears to be a first wave of migration (Erickson and Goins, 1995). It was later discovered that bipotent precursor cells with the capacity to differentiate into either melanoblasts or Schwann cells migrate along developing nerve sheaths in a second wave to populate the limbs with melanocytes (Adameyko et al., 2009). In cells migrating along the dorsolateral pathway, the expression of lineage-specific markers such as MITF and melanogenic enzyme dopachrome tautomerase (DCT) occurs around E10.5 (Larue et al., 2013; Mort et al., 2015).

By E11.5, melanoblasts begin to cross the basement membrane from the dermis to invade the epidermis. Their migration and invasion relies on several cytoskeletal organization factors such as RAC1 (Li et al., 2011; Mort et al., 2015). Outside of intrinsic migratory factors, melanoblasts presumably require external guidance cues to find the epidermis, although the identity and timing of such factors are largely unknown. However, evidence from 3-dimensional culture systems suggests that the membrane-bound form of stem cell factor (SCF, also known as kit ligand, KITL) expressed in the basal layer of the developing epidermis may direct melanoblasts to localize at this
juncture (Tabone-Eglinger et al., 2012). While many melanoblasts cross into and expand in the epidermis, a subset of cells persist in the dermis.

As melanoblasts migrate throughout the developing embryo, they undergo massive proliferation (Larue et al., 2013). Again, WNT/β-catenin signaling is critical here, driving proliferation in both the epidermal and dermal compartments (Luciani et al., 2011). Lineage-specific ablation studies in vivo have revealed other key genes and pathways regulating melanoblast migration and proliferation, with mutant animals displaying loss of pigmentation and white spotting phenotypes. Endothelin 3 (EDN3) is expressed in mesenchymal cells and signals through endothelin receptor-B (EDNRB) on melanoblasts, driving their proliferation in the dermis as well as their migration (Larue et al., 2013; Lee et al., 2003; Shin et al., 1999). SCF/c-KIT signaling also plays an essential role in proliferation, as well as in cell survival and migration (Yoshida et al., 1996a, Yoshida et al., 1996b). This crucial ligand is expressed by the mesenchyme and epidermis during embryonic development, driving melanoblast expansion specifically once cells reach the epidermis.

By E15.5, melanoblasts cluster around developing hair follicles (HFs) and sweat glands through a process that is still poorly characterized. There is some evidence that the chemokine receptor CXCR4 expressed on melanoblasts plays a role in their positioning, as disruption of this receptor in vivo results in melanoblasts aggregating abnormally in the epidermis. In culture, the CXCR4 ligand SDF-1 functions as a chemoattractant (Belmadani et al., 2009). Experiments using Dct-LacZ embryonic skin explants and ligand-coated beads demonstrated that while SCF serves as a chemokinetic factor facilitating melanoblast migration, it does not act as an attractive force (Jordan and Jackson, 2000). However, given the complex dynamics of morphogenesis, it is likely
that a plethora of other factors in addition to SDF-1 and SCF instruct melanoblast localization to their target destinations.

Once at the HFs, these progenitors migrate towards the base of the down-growing hair bulb and continue to differentiate into pigment-producing melanocytes that supply melanin for the first round of hair growth. However, a fraction of the migrating melanoblasts associate with the mid-portion of the HF in a specialized region known as the bulge where hair follicle stem cells (HFSCs) reside. Here, melanoblasts are maintained in an immature and quiescent state. It is still not understood how these colonizing melanoblasts initially become segregated into two compartments of bulge-associated immature and hair bulb matrix-associated differentiating cells.

The major localization of melanocytes is the skin, where they reside in both HFs and the basal layer of the epidermis. However, melanocytes are found in other organs, including the eye, inner ear, brain, and heart. While their primary purpose is to generate melanin pigment for UV protection, their unexpected locations in other sites throughout the body seem to suggest that melanocytes may have surprising auxiliary functions. While melanocytes in the kidney marrow of fish shield hematopoietic stem and progenitor cells from UV damage (Kapp et al., 2018), analogous to their role in the skin, conversely, melanocytes in the ear are actually critical for hearing and are thought to modulate the chemical composition of endolymph (Price and Fisher, 2001). Future studies on these unconventional melanocyte-lineage populations may lend further insight into other potential roles these cells play in the skin.

1.2 Tissue melanocyte stem cells (McSCs) and their progeny in the HF

Adult tissue-specific stem cells (SCs) are populations of long-lived cells that maintain and regenerate tissues in homeostasis and injury over the course of an organism’s lifetime. For many
decades, researchers posited that a pool of SCs must exist in order to replenish the melanocytes of the HF, which are eliminated at the end of each hair cycle (Nishimura, 2011). Furthermore, so-called “amelanotic melanocytes” were observed in the ORS of human HFs (Staricco, 1959). In 2002, Nishimura et al. demonstrated that the melanoblasts that initially localize to the bulge region fulfill the criteria for tissue SCs. These Dct-LacZ+ cells remained immature and unpigmented through adulthood and were slow-cycling, as demonstrated by their ability to retain bromodeoxyuridine (BrdU) in pulse-chase experiments. After depletion of their differentiating progeny with KIT blocking antibody, these immature cells in the bulge survived and were able to both self-renew and repopulate the hair bulb compartment with differentiating melanocytes in the subsequent hair cycle. In hair reconstitution assays using whisker follicle fragments, only implants with the lower permanent portion of HFs (where these undifferentiated Dct-LacZ+ cells were localized) gave rise to pigmented hair in albino animals. This population of cells are hence referred to as melanocyte stem cells (McSCs) (Nishimura et al., 2002; Nishimura, 2011)

To achieve growth of pigmented hair, HFSCs, which fuel the growth and regeneration of the HF, and McSCs must coordinate their behavior. Hair growth occurs through stages of rest, active growth, and regression (Fig. 1.1) (Gonzales and Fuchs, 2017). In telogen, hair follicles are at rest, with both HFSCs and neighboring McSCs in quiescence. At the onset of anagen, or active hair growth, HFSCs at the base of the bulge and nearby McSCs become activated to proliferate. HFSCs of the hair germ (HG) give rise to short-lived proliferative progeny that envelope the mesenchymal dermal papilla (DP), forming a hair bulb of matrix cells. HFSCs in the bulge generate the downward growing outer root sheath (ORS), creating distance from the proliferating hair bulb and DP and restoring quiescence to the bulge and upper ORS compartments (Hsu et al., 2014; Yang et al., 2017). Similarly, in early anagen, activated McSCs (aMcSCs) enter a brief
window of proliferation to give rise to committed, proliferative progeny (McCP). The McCP become spatially segregated from the SC compartment and associate with the DP and hair matrix. Here in the hair bulb, McCP continue to differentiate into melanocytes that produce and transfer pigment to neighboring hair cells, imparting color to the growing hair. Finally, in catagen, the destructive phase of the hair cycle, mature melanocytes undergo apoptosis alongside the lower two thirds of the follicle (Tobin et al., 1998).

Figure 1.1 McSCs undergo periodic bouts of quiescence, activation, and differentiation throughout the hair cycle.

Hair cycling in mice is highly predictable and can be easily tracked and manipulated through method such as hair plucking, which induces anagen. Given that the tissue SC populations residing in the HF exhibit periodic bouts of activity in sync with cycling, the mouse skin is an excellent system with which to temporally investigate SC quiescence (reversible cell cycle exit), activation (proliferation), lineage commitment, and differentiation in vivo. While HFSCs have been thoroughly studied and profiled in this context, less is known about the dynamic behavior of
McSCs. Here, mouse models present an additional advantage, because unlike human skin, mouse back skin lacks epidermal melanocytes. Unlike the melanocyte progeny generated by McSCs in the HF, epidermal melanocytes divide infrequently and produce and transfer melanin depending on the skin’s UV exposure (Davis et al., 2019). Therefore, the mouse skin provides a simplified system in which to specifically probe McSCs and their lineage. A more complete understanding of melanocyte lineage progression under homeostatic conditions is a critical prerequisite to achieving greater insight into melanocyte-related pathologies like melanoma and eventually developing novel therapeutic approaches for such conditions.

1.3 Key regulators of McSC quiescence, activation, and melanocyte differentiation

Tissue SCs protect their long term potential through precisely controlling their transitions between quiescence, activation, and differentiation. Like Muscle SCs, hematopoietic SCs, neural SCs, and HFSCs, McSCs exhibit periodic activity (van Velthoven and Rando, 2019). Dysregulation of the cellular states of SCs occurs during aging and can contribute to declining tissue repair and fitness, as well as in disease states characterized by excessive cell proliferation, such as cancer. Regulation of these states and the transitions between them occurs at both the cell intrinsic and extrinsic levels.

Lineage and cell state-specific TF expression is a defining feature of cell-intrinsic regulation. Master regulator MITF directs diverse cellular processes at all stages of development, including survival, proliferation, and melanocyte differentiation (Goding and Arnheiter, 2019). It coordinates these myriad functions by acting differently at low, medium, and high levels (Carreira et al., 2006), as well as by interacting with other TFs (Goding and Arnheiter, 2019). Its expression is regulated by the TFs PAX3 and SOX10 (Lang et al., 2005; Harris et al., 2013; Potterf et al.,
In McSCs, PAX3 is thought to both restrict lineage potential and prevent differentiation (Lang et al., 2005). SOX10 is necessary for McSC maintenance, but if levels are too high, SCs prematurely differentiate and are lost (Harris et al., 2013). In addition to these critical TFs, expression of the anti-apoptotic protein BCL2 is necessary for McSC maintenance and is especially critical as cells return to quiescence (Nishimura et al., 2005). During McSC activation, B-RAF and C-RAF kinases together are essential for cell cycle entry (Valluet et al., 2012).

Ultimately, the role of the niche and the extrinsic cues it provides are indispensable for the appropriate regulation of McSC activity, converging on these TFs to control cellular behavior. Functional studies in mice have demonstrated that neighboring HFSCs to which McSCs adhere are a crucial component of their specialized microenvironment. HFSCs express the hemidesmosomal transmembrane collagen, COL17A1, which is essential for their maintenance, quiescence, and expression of transforming growth factor beta (TGF-β) (Tanimura et al., 2011). While COL17A1 is essential for the structural integrity of the McSC niche, TGF-β from HFSCs promotes McSC quiescence (Nishimura et al., 2010; Tanimura et al., 2011). Cell-cell interactions are again important for Notch signaling, which is necessary for McSC maintenance (Moriyama et al., 2006; Schouwey et al., 2007). At the same time, Notch signaling through RBP-J in HFSCs regulates levels of retinoic acid, changes in which sensitize McSCs to differentiation (Lu et al., 2020).

At the onset of anagen, WNT/β-catenin signaling activates both HFSCs and McSCs. Additional crosstalk occurs here, as activated HFSCs secrete EDN-1 which further induces McSC proliferation (Rabbani et al., 2011; Takeo et al., 2016). Loss of the TF NFIB in HFSCs leads to expression of EDN-2, causing expansion of McSCs and precocious differentiation (Chang et al., 2013). As anagen progresses, HF lineages continue to guide McSC differentiation. WNT/β-catenin
signaling continue to be essential, as mice lacking β-catenin in the melanocyte lineage show reduced proliferation of McSCs in the bulge as well as in their progeny in the hair bulb, which fail to fully differentiate (Rabbani et al., 2011). Proliferating and differentiating lineage cells (McCP) express c-KIT receptor, and overexpression of its ligand stem cell factor (SCF) increases the number of melanocytes in the hair bulb. Conversely, interrupting this signaling cascade with c-KIT blocking antibody induces hair graying due to reduced melanocytes and impaired differentiation (Botchkareva et al., 2001). In their landmark study identifying McSCs, Nishimura et al. (2002) employed this blocking antibody to show that McSCs persist and replenish differentiating progeny in subsequent hair cycles. Later, Krox20-expressing hair shaft progenitors in the matrix were found to be a key source of essential SCF that fuels melanocyte differentiation (Liao et al., 2017).

However, HFSCs and their lineage are not the only niche cell types that influence McSC activity. Groundbreaking work by Zhang et al. (2020) investigating the link between stress and hair graying revealed the influence of sympathetic nerves on McSC activity. qMcSCs express ADRB2, which receive stress signals from nerves via the neurotransmitter norepinephrine, inducing proliferation, migration, and differentiation out of the bulge niche. The mesenchymal DP of the HF is also a key regulator and source of SCF (Yoshida et al., 1996). Wnt/β-catenin activity in the DP directs pigment-type production in melanocytes via agouti signaling protein (ASIP) or agouti-inhibitor corin expression. Melanocortin 1 receptor (MC1R) is expressed on melanocyte lineage cells and binds either alpha-melanocyte-stimulating hormone α-MSH or ASIP to induce eumelanin or pheomelanin production, respectively, via cAMP activity (Enshell-Seijffers et al., 2010).
The melanocyte lineage can also be regulated on a systemic level. Hormones like proopiomelanocortin (POMC)-derived neuropeptides adrenocorticotropic hormone (ACTH) and several MSHs are produced in the pituitary gland and can induce pigment production in melanocytes of the skin (Ito et al., 2005; Suzuki et al., 1996). Interestingly, a form of epidermal hyperpigmentation known as melasma can be triggered by fluctuations in sex hormones from pregnancy or medication (Filoni et al., 2019). There is also evidence of an emerging role of McSC crosstalk with the innate immune system via MITF (Harris et al., 2018).

Throughout the animal kingdom, regulators of the McSC lineage are manipulated to achieve hair color variations and patterns with functions beyond UV protection, including camouflage, inter- and intra-species signaling, mating rituals, and thermoregulation. Different colors can be achieved through loss of key components of pigment production (i.e. mutation of Tyr in CD-1 mice, resulting in albinism), or through more global mechanisms impacting the McSC differentiation trajectory. For example, the back stripe pattern of the African mouse *Rhabdomys pumilio* is achieved through differential expression of the TF *Alx3*, which suppresses MITF-induced differentiation to produce regions with lighter hair (Mallarino et al., 2016). Differential regulation and timing of SC activity and differentiation to achieve patterning has also been demonstrated in birds, where nonpigmented melanocyte progenitors in the lower bulge region of feather follicles periodically give rise to transit-amplifying progeny that differentiate as they move upward into the so-called barb region (Lin et al., 2013).

### 1.4 Dysfunction in McSCs and their lineage in aging and disease

As is the case for other tissue SC types throughout the body, dysfunction in how and when McSCs undergo these cell state transitions occurs in aging and can result in disease. Interest in
correcting abnormalities in this lineage stem from cosmetic to life-saving motivations. Over the course of aging, humans exhibit hair graying and also areas of skin hyper- and hypopigmentation. This reduced pigmentation can compromise UV protection, contributing to increased damage and decline of the skin tissue overall. With advanced age, the prevalence of melanoma also increases. Melanoma is the deadliest form of skin cancer, with cutaneous cases accounting for 55,500 deaths per year, as of 2018 (Schadendorf et al., 2018). Therefore, understanding how McSCs and their melanocyte lineage can go awry in these contexts is important not only for SC biology, but also for combatting serious clinical issues.

1.4.1 Natural mechanisms of McSC depletion

Dysfunction in the McSC lineage in the HF results in hair graying (also known as canities), one of the most ubiquitous and visible signs of aging (Nishimura, 2011). Gray or white hair phenotypes in mice can occur through two major routes: 1) dysfunction or loss of cells in the McSC compartment, or 2) in the differentiating progeny (Fig. 1.2 A-B). Some have posited that age-related hair graying is caused by oxidative stress in melanocytes of the hair bulb, where reactive byproducts of melanin biosynthesis damage cells over time and lead to their eventual loss (Wood et al., 2009). However, Nishimura et al. (2005) demonstrated that in both humans and mice, age-related hair graying can result from loss of McSCs. Without the bulge SC reservoir, during active hair growth, fewer or no pigment-producing progeny can be generated, leading to irreversible loss of hair color.
Figure 1.2 Loss of hair color can occur through defects in McSCs or their progeny. (A) Loss or dysfunction in McSCs results in the generation of fewer or no differentiating progeny to make pigment. (B) Alternatively, problems downstream in the differentiating progeny themselves can result in reduced pigment production and/or transfer.

There are several causes and mechanisms through which McSCs can be depleted. The accumulation of genotoxic damage in qMcSCs can lead to ectopic differentiation in the bulge and compromised self-renewal, and mice lacking ataxia-telangiectasia mutated (ATM) kinase are sensitized to McSC differentiation upon ionizing radiation (Inomata et al., 2009; Ueno et al., 2014). Indeed, individuals with progeria syndromes have impaired DNA damage response mechanisms and often show premature hair graying (Nishimura, 2011). During acute stress, experiences of
which accumulate over an organism’s lifetime, norepinephrine is secreted by sympathetic nerves and induces McSC proliferation and migration to the epidermis, depleting the bulge population and resulting in gray hair (Zhang et al., 2020). At the same time, the aging HF niche and epidermis can compromise McSCs as well as differentiated melanocytes. In human and mouse skin, COL17A1 levels are reduced with age, leading to McSC differentiation in HFs and loss of epidermal melanocytes (Liu et al., 2019; Matsumura et al., 2016; Tanimura et al., 2011).

On a more local scale, McSCs can be affected by insults to the skin such as wounding, UV exposure, and inflammation. Dermatologists and patients have long noticed the presence of hyperpigmentation around wounded and scarred areas of the skin. While epidermal melanocytes are likely also affected here, Chou et al. (2013) demonstrated that after excisional wounding and UVB irradiation in mice, McSCs migrate out of the HF niche. They do so without self-renewing, moving upward into the epidermis where they differentiate into melanocytes and are thought to provide additional UV protection to vulnerable damaged cells during wound healing. This phenomenon results in white hairs in the wound area due to permanent McSC depletion from HFs. UVB-induced McSC migration is in fact the basis of a form of re-pigmentation phototherapy used to treat vitiligo, a skin condition characterized by depigmented patches of skin due to loss of epidermal melanocytes (Nishimura, 2011). Multiple causes of melanocyte destruction have been proposed, from cell detachment to oxidative stress, but the prevailing notion is that it occurs through autoimmune attack (Bergqvist and Ezzedine, 2020). Follicular McSCs appear to be spared from these assaults and can therefore be stimulated by UV light therapy to migrate to the epidermis and differentiate into melanocytes to replenish skin pigmentation. A recent study has provided new insights into how the melanocyte lineage normally defends itself against the immune system. McSCs from Mitf heterozygous mice were found to express higher levels of type I interferon genes
relative to control. Curiously, when viral mimics were introduced into Sox10 overexpressing mice to raise an innate immune response, hair graying was induced through an unknown mechanism (Harris et al., 2018). These findings indicate that MITF plays a role in repressing interferon signature genes normally, and in certain circumstances, a heightened immune response can contribute to hair graying.

Finally, there is currently scant evidence for the how autophagy, a self-degradation process whereby cells clear damaged organelles or proteins, affects McSCs. However, given its role in protecting against oxidative stress, which is increased by melanin production, and protecting the integrity of other long-lived tissue and quiescent SCs (van Velthoven and Rando, 2019), disrupted autophagy is a plausible route through which McSCs might become compromised in aging. As the body’s first line of defense, the skin will encounter various challenges over the course of an organism’s lifetime. It is likely that many if not all of the mechanisms discussed culminate to contribute to the overall depletion of McSCs and dysfunction of their melanocyte progeny.

1.4.2 Melanoma and its SC properties

On the other end of the spectrum is cancer, a pathologic shift towards overactivation and unrestrained proliferation. Melanoma is a highly aggressive form of skin cancer prone to metastatic spread. It can occur in cutaneous, acral (on the palms of hands, soles of feet, or nailbeds), mucosal, uveal, and conjunctival forms. Common oncogenic driver mutations include \(BRAF^{V600E}\), \(NRAS\), \(NFI\), and genes in cell cycle regulation, with loss-of-function mutations in tumor suppressors genes like \(P53\) and \(PTEN\) (Schadendorf et al., 2018). Such mutations induce overactivation of several pathways like MAPK and PI3K signaling. Different melanomas exhibit distinct mutational signatures and molecular features (Trucco et al., 2019), depending on body location, disease stage progression, and complex interactions between the tumor and its microenvironment and the
immune system. For instance, UV-induced cutaneous melanomas exhibit a high mutational burden characterized by $\text{C}\rightarrow\text{T}$ and $\text{G}\rightarrow\text{T}$ mutations, while non-UV induced acral and mucosal melanomas often have fewer mutations but higher incidence of structural variants like copy number variation. (Alexandrov et al., 2013; Hayward et al., 2017; Schadendorf et al., 2018; Trucco et al., 2019). As primary lesions progress, they are prone to metastasize through the lymphatics (Ubellacker et al., 2020) to the lymph nodes and then to the blood circulation to disseminate throughout the body. Once a patient has progressed to metastatic disease, prognosis and survival outcomes are significantly worse (Davis et al., 2019).

These cancer cells exhibit a high degree of plasticity and are able to undergo “phenotype switching” to oscillate between more or less proliferative and or differentiated states, which is often dictated by levels of MITF (Carreira et al., 2006; Cheli et al., 2011). This agility can be exploited to change behavior throughout melanoma progression (i.e. proliferative cancer cells in the tumor switching to a more stem-like state during invasion) and may in some cases confer drug resistance. For example, in response to inflammatory cytokines released by T cells, melanomas can dedifferentiate and downregulate their expression of melanocytic antigens in order to evade immunotherapy (Landsberg et al., 2012). Another aspect of this plasticity centers on heterogeneity within the tumor (Tirosh et al., 2016) and the concept of a subpopulation of “melanoma stem cells” endowed with unique properties that allow them to self-renew and both sustain and repopulate tumors (Schatton et al., 2008). These cells may be identified by expression of distinct markers, and although often slow-cycling, drive tumor growth through supplying proliferative progeny (Boiko et al., 2010; Fang et al., 2005; Roesch et al., 2010).

A popular hypothesis that has been proposed to explain the deadly propensities of melanoma for plasticity and metastasis is that transformed cells are able to re-access their neural
crest and or SC programs. The capacities of melanoblasts for exponential proliferation and impressive migration and colonization during embryogenesis are tightly regulated, but if transformed cells could tap into these developmental mechanisms without proper restraints, the results would be disastrous. Gupta et al. (2005) demonstrated that when transformed with the same oncogenes, melanocytes give rise to melanomas that metastasize, but fibroblasts and mammary epithelial cells produce tumors that rarely lead to metastatic disease. These findings point to the importance of the melanocyte lineage cellular context in endowing these aggressive capabilities. Studies in zebrafish have been particularly illuminating here, highlighting the neural crest features that underlie melanoma initiation, invasion, and drug resistance (Fazio et al., 2021; Johansson et al., 2020; Kaufman et al., 2016; Santoriello et al., 2020). For example, a key study by Kaufman et al. (2016) employed a crestin-eGFP reporter to demonstrate that p53 and BRAF mutant cells induce a neural crest gene signature during melanoma initiation (Kaufman et al., 2016). Similarly, global embryonic melanoblast transcriptomic data has recently been mined to uncover novel regulators of melanoma metastasis (Marie et al., 2020).

Given the striking parallels between melanomas and their neural crest and melanoblast precursors, the field has debated whether adult McSCs in HFs and sweat glands can act as the melanoma cell-of-origin. Indeed, McSCs not only give rise to proliferative progeny but retain migratory capacity, abilities that in a malignant context are akin to fueling tumor expansion and metastasizing, respectively. McSC quiescence is also interesting here, as melanoma cells that have metastasized are able to exist in a state of dormancy for years in distal sites of the body before being reactivated to generate a metastatic tumor. Curiously, immunocompromised patients can develop melanoma after receiving organ transplants, suggesting that their immunocompetent donors had dormant cancer cells lurking in that organ, but appeared healthy otherwise (Strauss and
Thomas, 2010). Furthermore, there are melanotic (pigmented) and amelanotic (unpigmented) forms of melanoma, which seems to suggest the possibility of both melanocyte (pigmented) and McSC (unpigmented) precursor cells.

Several recent studies have sought to tackle this controversial cell-of-origin question using mouse models. Using TyrCreER to drive mutant Braf\textsuperscript{V600E} and Pten loss-of-function in McSCs and their lineage, Moon et al. (2017) demonstrated that McSCs could give rise to melanoma, but to do so required their activation. Upon UV exposure and inflammation, transformed McSCs migrated to the epidermis where they expanded and generated down-growing melanomas. The authors claim that SC quiescence is tumor suppressive in this context. In a back-to-back report, Köhler et al. (2017) approached a similar question using this mouse model, but focused on the tail, where amelanotic and mature epidermal melanocytes reside (Glover et al., 2015). Their evidence suggests that melanocytes can give rise to melanomas, but that they undergo transcriptional reprogramming to dedifferentiate during this process. Analogous to the results of Moon et al. in qMcSCs, the quiescent amelanotic population of cells in the tail appear refractory to melanomagenesis. Later, using a new c-Kit-\textit{CreER} driver to more specifically transform the SC compartment, Sun et al. (2019) further substantiated and extended evidence suggesting that McSCs can serve as the melanoma cell-of-origin. Outside of cutaneous skin, the palms and soles of feet lack HFss but are also common sites for melanoma. Given their ability to divide and migrate to the epidermis upon oncogenic stress, the immature and slow-cycling lineage cells that reside in the secretory portion of sweat glands are intriguing candidates for acral melanoma tumor-initiators (Okamoto et al., 2014). At this point, there is compelling evidence in mice that melanomas can originate from either McSCs or their progeny, but whether this actually occurs in human cancers remains to be definitively demonstrated.
1.5 Summary

Melanoblasts, tissue McSCs, and their melanocyte progeny have been an attractive model system with which to approach myriad questions, ranging from those pertaining to basic genetics to embryonic development to mechanisms of migration and proliferation to organelle biogenesis and trafficking. In my thesis research, I have harnessed the tractable model of the mouse skin and HF to elucidate the molecular mechanisms that underlie McSC quiescence, their transition to activation and proliferation, and their complete differentiation into pigment-producing cells. A more thorough understanding of this lineage and how it functions normally in vivo is a critical parallel to studies being conducted in abnormal cell states like melanoma and vitiligo. Such studies may not only inform our understanding of disease, but may have broader implications across other types of adult tissue SCs, especially those that cycle between quiescent and activated states to generate transit amplifying progeny in response to periodic tissue demands. For the McSCs and their lineage, there is still much to learn about how the differentiation process is accomplished, especially with regard to the intermediate transit-amplifying stages and how different signaling inputs are integrated. To unearth new insights, I sought temporal and single cell-level information about transcription and chromatin states, prioritizing experiments that could be performed in the native mammalian tissue context.
CHAPTER 2:
ELUCIDATING THE TRANSCRIPTIONAL DYNAMICS OF MELANOCYTE LINEAGE PROGRESSION THROUGH STEM CELL ACTIVATION AND DIFFERENTIATION
2.1 Introduction

McSCs move from quiescence to activation and differentiation in step with hair cycle progression. Given its predictability and ease of manipulation, the murine HF is an attractive model system with which to interrogate McSC behavior. While elegant functional studies in mice have yielded important discoveries regarding the specific pathways involved in McSC lineage progression, unbiased approaches like high throughput sequencing provide an opportunity to uncover novel insights about complex biological processes. Previous studies have used single-cell qPCR of qMcSCs, melanocytes, and embryonic melanoblasts (Osawa et al. 2005), bulk RNA-sequencing of McSCs (Joshi et al., 2019; Moon et al., 2017; Zhang et al., 2020) or single-cell comparisons of McSCs and melanoma using 10X Genomics (Sun et al., 2019).

In my investigation of this lineage, I also wanted to start with a more global vantage point with which I could establish my own datasets and address gaps in our understanding. Broadly, I hypothesized that distinct transcriptional programs fueled by specific TFs drive quiescence, activation, and differentiation. In my approach, I wanted to have single cell resolution that would allow me to delineate the intermediate and likely heterogeneous activation and differentiation steps. To best accomplish this, I also needed a sequencing method that would afford high depth of coverage to discern maximal transcriptional differences between these related cell types.

To this end, I implemented transgenic mice expressing fluorescent proteins in the melanocyte lineage and characterized the activity and localization of McSCs and their progeny throughout hair cycling. I then designed fluorescence-activated cell sorting (FACS) strategies to isolate lineage cells from the skin at three discrete time points and performed single cell RNA-seq (scRNA-seq) with Smart-Seq2 (Picelli et al., 2013; Picelli et al., 2014). With this approach, I
obtained high quality transcriptional data with which to perform comparative gene expression and pathway analyses, illuminating both expected and novel insights into this differentiation process.

2.2 Results

2.2.1 Characterizing McSC lineage activity during hair cycling in the mouse skin

To facilitate the visualization and FACS-based isolation of McSCs and their progeny, I took advantage of the Dct-eGFP mouse strain (Gong et al., 2003). DCT is a melanogenic enzyme and ubiquitous lineage marker expressed by approximately E10.5. in mice (Mort et al., 2015). Using immunofluorescence (IMF) analysis of the skin, I confirmed that eGFP expression overlapped with endogenous DCT and was present in both McSCs in the bulge region and in their differentiating progeny in the anagen hair bulb (Fig. 2.1 A). In second telogen, or approximately postnatal day 60 (P60), qMcSCs in the bulge region were the only melanocyte lineage present in the skin (Fig 2.1 A, left). To assess the timing of peak McSC proliferation and enable isolation of aMcSCs, I measured incorporation of 5-ethynyl-2-deoxyuridine (EdU) within a 24 hour pulse at time points spanning from late first telogen through early anagen progression (P20-23) in Dct-eGFP mice (Fig. 2.1 B-C). Quantification of EdU+ endogenous DCT+ cells indicated that McSC proliferative activity peaked during anagen I-II, which occurred at P21 in males of this background strain (Fig. 2.1 C-D). At this point, morphologically, HG expansion and DP engulfment was minimal in HFs, and the majority of aMcSCs had not yet become spatially segregated from the bulge region. Later on in anagen III, HGs had developed into hair bulbs distanced from the bulge, which allowed for evaluation McCP behavior during differentiation (Fig. 2.1 C, E). At P23, approximately 30% of DCT+ cells in the hair bulbs were proliferating, suggesting continued expansion of progeny after initial McSC activation (Fig. 2.1 F).
Figure 2.1 Characterization of McSC activity and differentiation into melanocytes throughout the hair cycle reveals time points at which to capture different cellular states. (A) IMF images of qMcSCs (left, 2nd telogen (telo) ~P60), aMcSCs (middle, anagen II (ana II) at P21), and hair bulb McSC progeny (right, anagen VI at P10); red dashed box indicates enlarged areas below. (B) Experimental schematic used to assess McSC and McCP proliferation throughout anagen. (C) McSC activation generates hair bulb-associated McCPs. (D) Bulge/HG McSC proliferation. (E) Percent of HFs with hair bulb McCPs. (F) Hair bulb McCP proliferation. [P20 n=53 HFs; P21 n=63 HFs; P22 n=36 HFs; P23 n=48 HFs (P20 n=1 mouse; P21-23 n=2 mice)]. For all IMF, scale bars=25 µm, white dashed line outlines HFs at the epithelial-mesenchymal junction, Bu=bulge.
By full anagen (anagen VI), differentiating pigment-producing melanocyte progeny were present in the hair bulb. However, both McSCs and their progeny express Dct-eGFP, so to distinguish these differentiating cells from their SCs using FACS, an additional fluorescent marker was necessary. For this purpose, I crossed Dct-eGFP mice to lymphoid enhancer factor 1(Lef1)-RFP mice that express RFP in the WNT-responsive differentiating melanocytes but not in McSCs (Fig. 2.1 A, right) (Rendl et al., 2005). In full anagen hair bulbs at P10, Lef1-RFP was expressed in melanocyte lineage cells and the DP, but melanocytes could be distinguished by co-expression of Dct-eGFP. By characterizing McSC lineage activity in the mouse skin, I could then use these time points and fluorescent proteins as the basis upon which to develop cell isolation protocols.

2.2.2 McSCs can be purified from mouse skin

Having established the optimal time points and fluorescent markers with which to label qMcSCs, aMcSCs, and their differentiating progeny (including mature melanocytes), I next designed FACS strategies to isolate these populations. Using well-established protocols from the Fuchs Lab, I mechanically and chemically digested dissected mouse back skin according to the hair cycle stage to generate a single cell suspension (Fig. 2.2 A). For all purification schemes, I used antibodies against cell surface markers of contaminating cell types in the skin, including endothelial (CD31+), immune (CD45+), fibroblast, platelet, and adipocyte (CD140a+), epidermal and HF infundibulum (Sca-1+) and bulge HFSC (CD34+) cells. After gating against these cell types, qMcSCs from second telogen skin were defined as Dct-eGFP+ integrin α-6medium-low cells; in anagen I-II (P21), I collected Dct-eGFP+ integrin α-6all cells, in case changes in integrin expression occurred during this dynamic and heterogeneous activation state (Fig. 2.2 B, top). Finally, in anagen VI (P10), differentiating lineage cells were identified as Dct-eGFP+, Lef1-RFP+, and c-KIT (CD117)+ cells (Fig. 2.2 B, bottom).
For initial validation of these strategies, I used RT-qPCR followed by bulk RNA-sequencing of the “bookend” populations of telogen qMcSCs and anagen VI differentiating McSC progeny. These RNA-seq data showed high reproducibility between biological replicates, and compared to published HFSC RNA-seq data, there was robust expression of known lineage markers including *Dct*, *Sox10*, and *c-Kit* (Fig. 2.2 C). Differential gene expression analysis with DESeq2 (Love et al., 2014) revealed thousands of significant transcriptional differences between qMcSCs and their differentiating progeny (2,385 genes up in qMcSCs; 2,819 up in differentiating progeny, padj≤0.001, log2fold≥1) (Fig. 2.2 D; see also Table S1 of Infarinato et al., 2020). Notably, these included several previously described population-specific gene expression differences, including *Bcl2* in qMcSCs and *Oca2* and *Mc1r* in the differentiating progeny.
Figure 2.2 McSCs and their differentiating progeny can be isolated from mouse skin. (A) Experimental approach used to isolate McSC lineage and perform transcriptional profiling. (B) FACS purification schemes for McSCs (top) and differentiating hair bulb McSC progeny (bottom). (C) Validation of sorting strategies by bulk RNA-seq. Bar graph shows Transcripts Per Million (TPM) mean values relative to HFSCs. Error bars represent standard deviation (HFSCs n=2, qMcSCs n=2, hair bulb McSC progeny n=3). (D) Mean average plot of differentially expressed genes between qMcSCs (negative fold change) and their differentiating progeny (positive fold change). (E) Correlation matrix illustrating Euclidean distances between biological replicates (prog.=differentiating McSC progeny).

While these data provided a solid foundation for my study and showed good correlation between biological replicates (Fig. 2.2 E), I suspected that even in these carefully purified populations, there might be transcriptional heterogeneity due to intermediate transition steps in the lineage and asynchrony of cells passing through each stage, as well as some degree of contamination from other cell populations in the skin. The ability to detect these differences would
be especially critical at the activation and transit-amplifying steps, where my IMF analyses indicated the presence of non-cycling and cycling McSCs and early McCP populations in the hair bulb (Fig. 2.1 C-F). To both capture this heterogeneity and ensure high purity, we performed scRNA-seq with Smart-Seq2, which provides higher depth of coverage than Drop-seq or 10X Genomics (Picelli et al., 2013) on telogen qMcSCs, anagen I-II aMcSCs, and anagen VI differentiating progeny (Fig. 2.2 A). To minimize the capture of non-lineage cells for this purpose, I also implemented more stringent FACS gating.

2.2.3 McSCs and their progeny exhibit distinct transcriptional signatures throughout lineage progression in vivo

Single cell mRNA libraries were prepared according to the Smart-Seq2 protocol (Picelli et al., 2013; Picelli et al., 2014) and subjected to sequencing. Prior to comparative analyses, a variety of quality control tests and filtering parameters were performed (Fig. 2.3). External RNA (ERCC) spike-ins were implemented to discern technical noise associated with library preparation and sequencing from biological variability (Fig. 2.3 A) (Brennecke et al., 2013). To eliminate low quality libraries from further analysis, cells with transcript reads from <1,250 genes were removed. Any \( Dcr^{low} \) (logTPM+1<6) or \( Krt15^{+} \) (logTPM+1>6) cells were assumed to be non-melanocyte lineage cells and were also eliminated from further analysis. The retained cells had an average of 1x10^5 reads/cell, with few reads from the full datasets mapping to the mitochondrial genome, suggesting good cell viability (Fig. 2.3 B). Libraries were processed in multiple rounds of sequencing, so to determine the impact of batch effect, cells were visualized according the biological replicate (mouse) from which they originated (Fig. 2.3 C, left and middle). McSCs from telogen and anagen I-II exhibited strongly overlapping profiles; for anagen VI differentiating progeny, where slight variations in age and rapidly progressing hair cycle stage might influence
transcriptional profiles, cells from individual mice were still most closely related to each other and showed a considerably degree of overlap. Finally, telogen qMcSCs displayed the least complex transcriptome (Fig. 2.3 B, middle and C, right), consistent with a previous report by Osawa et al. (2005) using single cell RT-qPCR. Furthermore, this low complexity transcriptome was also observed in a fraction of the anagen I-II McSCs, where a subset of cells are expected to remain quiescent during SC activation. These results suggest that reduced transcription is likely a genuine feature of qMcSCs, similar to what been described for several other types of quiescent SCs (van Velthoven and Rando, 2019).
Figure 2.3 scRNA-seq data from purified cells are of high quality. (A) Technical noise tests relative to ERCC spike-ins detects biologically variable genes for each population. (B) Quality control tests showing log number of counts, genes detected per cell, and percentage of mitochondrial reads for unfiltered datasets. (C) Unsupervised k-NN based clustering of melanocyte lineage cells colored by group identity (left), individual mouse (middle), and number of genes detected per cell (right).

Principle component analysis (PCA) with uniform manifold approximation and projection (UMAP) (Becht et al., 2019) coupled with k-Nearest Neighbors (k-NN) based community detection (Blondel et al., 2008) was used to visualize these datasets. Cells segregated into three distinct clusters, all of which showed robust expression of lineage markers such as Dct and Mitf (Fig. 2.4 A). As expected, clustering indicated that telogen and anagen I-II McSC populations (C1,
C2) were more similar to one another than to the anagen VI differentiating progeny (C3). To first understand what are the major transcriptional differences driving this separation and define features of stemness versus differentiation, differential gene expression and gene ontology (GO) analyses were performed on the combined cells of C1 and C2 versus C3 (Fig. 2.4 B; Table 2.1; see also Table S2 of Infarinato et al., 2020).

In this comparison, McSCs showed selective upregulation of transcripts for genes with known importance in stemness, such as Bcl2 (Nishimura et al., 2005) and Pax3 (Lang et al., 2005; Potterf et al., 2000). Unexpectedly, activator protein-1 (AP-1) family TFs (Fosb, Fos, Junb, Jun) featured prominently. At the same time, McSCs showed a striking upregulation of immunomodulatory genes (Socs3, Stat3, Stat5a, Il6st). Among these genes was Cd274, encoding programmed death-ligand 1 (PD-L1), an immune checkpoint protein that binds to PD1 receptor on T cells to inhibit their activation.

On the other side, the differentiating progeny of C3 showed robust upregulation of genes involved in melanogenesis including key melanin production enzymes (Tyrp1, Tyr) and melanosome proteins (Pmel, Gpr143, Oca2) and transporters (Rab38). Transcripts from well-known melanocyte differentiation pathways were also enriched, including SCF/c-KIT (Kitl) (Botchkareva et al., 2001; Liao et al., 2017), MC1R/α-MSH/ASIP (Mc1r) (Enshell-Seijffers et al., 2010), and WNT signaling (Lef1) (Rabbani et al., 2011). Consistent with their increased activity compared to SCs, differentiating progeny showed upregulation of several genes encoding enzymes involved in multiple forms of cellular metabolism as well as genes involved in semaphorin-plexin signaling (Plxna1/2/4, Sema6d).
Figure 2.4 McSCs and their progeny exhibit distinct transcriptional signatures. (A) UMAP representation and unsupervised k-NN based clustering of single cell data. Each dot represents a cell colored by its cluster (“C”) and known cell identity. For FACS cell identity, pink dots are telogen “qMcSCs” (n=104 cells, n=3 mice), gold are anagen I-II “aMcSCs” (n=193 cells, n=3 mice), and light blue are anagen VI “hair bulb McSC progeny” (n=308 cells, n=2 mice). Below, expression plots for the lineage markers, \textit{Dct} and \textit{Mitf}, across all three melanocyte populations. (B) Heatmap illustrating select differentially expressed genes between McSCs (C1 and C2) and McSC progeny (C3).

2.2.4 Transcriptional heterogeneity increases throughout differentiation

Having established the key transcriptional features that broadly define stemness and differentiation, I next turned to investigating the presence of intrapopulation heterogeneity, a concept that had received little attention in the McSC and melanocyte field. This was achieved through further sub-clustering of the original three clusters, maintaining conservative parameters so as to break down clusters based on the most prominent transcriptional differences. This analysis detected the presence of six distinct subpopulations and suggested that transcriptional heterogeneity increases as cells move from quiescence to activation and differentiation. C1
remained as one cluster, C2 split into C2a and C2b, and C3 broke into C3a, C3b, and C3c (Fig. 2.5 A).

To understand the features of these six sub-clusters, the stemness and differentiation signature genes in the initial comparison of C1 and C2 versus C3 were used to generate global scoring system (Fig. 2.5 B). Consistent with my IMF characterization of the lineage throughout the hair cycle (Fig. 2.1), these six sub-clusters could be delineated on the basis of differences in proliferation and melanogenesis gene expression. Therefore, a group of proliferation genes was also used to establish an overall score (Fig. 2.5 C, left). These analyses indicated that C1 had the highest stemness score, and C2 sub-clusters showed marked differences, where C2a was more stem-like than C2b. Consistently, a fraction of the C2b cells had positive differentiation scores, which were all high in the C3 clusters, especially in C3b and C3c. Global proliferation scoring provided further insight, suggesting that sub-cluster C2a was less quiescent than C1, but sub-clusters C2b and C3a were particularly enriched for cell cycling transcripts. Cell phase scoring provided further resolution, indicating signatures for G1/S, S, G2/M, and M stages (Fig. 2.5 C). Some cell cycle transcripts remained elevated in the more differentiated C3b and C3c sub-clusters, but overall, these were substantially lower than in C3a. At the same time, C3c expressed the highest levels of melanin biosynthetic and melanosome organization pathway genes, marking these as the most mature melanocytes in the dataset (Fig. 2.6 A-C). Based on these stemness, proliferation, and differentiation gene expression patterns, as well as the hair cycle stage at which they were isolated, it was possible to delineate these sub-clusters as qMcSCs (C1), aMcSCs (C2a), a transitional state from aMcSCs to early McCP (C2b), later McCP of the hair bulb (C3a), and differentiated melanocytes (C3b, c) (Fig. 2.6D). This provided high resolution with which to explore temporal changes in transcription throughout the differentiation trajectory.
Figure 2.5 Transcriptional heterogeneity increases throughout lineage progression. (A) Sub-clustering of each original cluster from Fig. 2.4 A. (B) Violin and UMAP plots depicting global stemness and differentiation scores throughout the six sub-clusters. (C) Violin plot of proliferation scores with cell cycle phase scoring.
Figure 2.6 Melanogenesis, cell cycling genes, and GO analysis further delineate sub-cluster identities. (A) Heatmap of select proliferation and melanogenesis mRNAs. (B) Expression plots of example proliferation genes and (C) melanogenesis genes. (D) Selected GO terms for each sub-cluster with False Discovery Rate (FDR).
2.2.5 Analysis of signaling pathways and biological processes associated with each sub-cluster

As a next step to understand the biological features of these distinct sub-clusters, GO analysis was implemented. Sub-clusters were compared against one another using differential gene expression analysis, and the resulting gene lists were subject to GO term processing using PANTHER (Mi et al., 2019; Thomas et al., 2006) and DAVID 6.8 (Huang et al., 2009a; Huang et al., 2009b) (Fig. 2.6 D, select PANTHER terms with FDR; see also Table S3-6 of Infarinato et al., 2020). When qMcSCs (C1) were compared to all anagen I-II McSCs/early McCPs (C2), expected pathways such as Notch (Moriyama et al., 2006) and TGF-βR signaling (Nishimura et al., 2010) were enriched. Genes and processes involved in “negative regulation of cell proliferation” (1.1E-7) and “autophagy” (p=1.7E-5) (DAVID 6.8) were also detected, consistent with the “common quiescence signature” that has been described for other types of tissue SCs (van Velthoven and Rando, 2019). In line with their transition to an activated state, C2 was dominated by cell division terms, and a few melanogenesis genes were significantly increased, including Oca2, Mc1r, and Rab32. To delineate the cellular processes that change as aMcSCs become early McCP, C2a was compared to C2b. This highlighted elevated nuclease activity and regulation of cell migration in C2a, while C2b showed enrichment of cell cycling and DNA replication and repair pathways, characteristic of a transit-amplifying cell population.

To dissect the differences between the three sub-clusters of McSC differentiating progeny in the anagen VI hair bulb, differential gene expression was performed on C3a late McCP versus C3c highly differentiated melanocytes and C3b melanocytes versus C3c. In both analyses, melanin biosynthesis and related processes comprised the top terms for C3c. C3a, the late McCP, showed upregulation of an intriguing number of cell signaling pathways, and C3b showed enrichment of cytoskeletal organization and cell division-related processes.
2.2.6 BMP and WNT signaling gene expression increases through differentiation

Turning to genes and pathways likely to be involved in lineage commitment and progression, I focused on cohorts of genes that robustly changed in expression during the less understood transitional stages of differentiation. Subdividing the McSC lineage into six transcriptionally distinct sub-clusters now revealed with higher resolution when specific signaling pathways became activated.

An interesting feature that became apparent was that WNT signaling and response were particularly elevated at the juncture between the anagen I-II C2b McCP and the anagen VI late McCP (C3a) in the hair bulb (Fig. 2.7 A, top). This marked transition was also reflected by the significantly enriched (FDR=1.7E-5) “canonical WNT signaling pathway” GO term in sub-cluster C3a when compared to C3c melanocytes (Fig. 2.6 D). Of further intrigue was a similar dramatic elevation of bone morphogenetic protein (BMP) pathway response genes at this same juncture (Fig. 2.7 A, bottom), and also the significant (FDR=1.5E-2) term “positive regulation of BMP signaling pathway” in the C3a McCP sub-cluster analysis (Fig. 2.6 D). Probing deeper into WNT pathway activation throughout the lineage revealed elevated levels of genes such as Lef1, Wif1, Mel and Wnt5a within the McCP/melanocyte populations (Fig. 2.7 B). Similarly, there was robust upregulation of BMP target genes Id1/2 and Msx1/2 and ligands Bmp2/7 in these differentiating populations (Fig. 2.7 C-D), consistent with their BMP receptor expression (Fig. 2.7 D-E).

WNT activity drives McSC expansion and downstream melanocyte differentiation (Rabbani et al., 2011). BMPs have been reported to elicit ligand and context-specific effects on melanin production in cultured cells (Bilodeau et al., 2001; Hsu et al., 2005; Jin et al., 2001; Kawakami et al., 2008; Park et al., 2009; Singh et al., 2012; Yaar et al., 2006; Yang et al., 2014). However, the role of this pathway in the native tissue microenvironment, as well as whether it
Figure 2.7 WNT and BMP signaling increase during melanocyte differentiation. (A) Violin plots of canonical WNT and BMP target gene pathway scores in each sub-cluster. (B) Expression plots of WNT and (C) BMP pathway genes. (D) Heatmap of BMP ligand, receptor, and transcriptional effectors over differentiation. (E) Expression plots of BMP receptors.

might engage with crosstalk with other niche signaling components was not well understood. Therefore, I endeavored to investigate the role of BMP signaling in melanocyte lineage progression in vivo and explore possible interplay with WNT. These studies are the subject of Chapter 3.
2.3 Discussion

In harnessing an unbiased approach and profiling cells from their native niche throughout lineage progression, this study represents a significant advance in our understanding of the transcriptional underpinnings of McSC quiescence, activation, and differentiation. It revealed the presence of six distinct transcriptional profiles, suggesting that the lineage increases in complexity as cells progress through differentiation. Further studies will be necessary to delineate whether these different profiles, especially those three sub-clusters in the differentiating C3 cluster, represent a continuum of states through which all cells pass, or perhaps also indicate differences in spatial organization within the HF and hair bulb niche, resulting in different extracellular cues and cell-cell adhesion. For example, cells in more direct contact with the DP might receive different levels of signals from the mesenchyme than those further out and closer to matrix TACs.

One significant argument for studying the dynamic developmental trajectory of this lineage is to gain insight into how normal features might be exploited or perturbed in disease. In this regard, several aspects of the McSC signature underscore the concept that melanomas harness SC programs. AP-1 factors regulate several cellular processes, but the particular upregulation in SCs was intriguing in light of their implications in cancer. For instance, in melanoma, c-JUN and MITF are thought to act antagonistically (Riesenbergs et al., 2015), and FOSL1 has been shown to induce melanocyte reprogramming and transformation, also downregulating MITF (Maurus et al., 2017). Given these striking parallels and the paucity of information regarding their role in the normal physiological context, AP-1 family TFs in the McSC lineage merit further study.

Furthermore, the upregulation of immune-related genes in McSCs might also suggest that the ability of melanoma to modulate and evade immune detection is rooted in a physiological SC property. Indeed, with regard to gaining potential insights into malignant traits, one major
advantage of studying adult tissue McSCs versus embryonic melanoblasts is understanding these SCs in the context of an immune-competent niche. Curiously, work by Riesenberg et al. (2015) suggest that dedifferentiated MITF$^{low}$ melanoma cells have higher expression of inflammatory genes. In this regard, the selective upregulation of $Cd274$ (encoding PD-L1) in McSCs in my dataset was particularly striking. PD-L1 is upregulated on melanoma cells to protect against immune attack, and targeting this interaction with immunotherapy has revolutionized melanoma treatment (Sharma and Allison, 2015). It is tempting to speculate that McSCs might harness molecules like PD-L1 to protect themselves from immune attack in situations when the protective HF niche might be compromised (i.e. wounding, inflammation) or during migration out of the follicle and into the epidermis. Indeed, McSC crosstalk with the immune system is an area of emerging study. Harris et al. (2018) demonstrated that MITF suppresses interferon signature genes. My data appear to be consistent, as qMcSCs with lower levels of MITF protein, show upregulation of many immune-related genes relative to their differentiating progeny, which accumulate nuclear MITF. Overall, these results add to the body of literature that report shared molecular features of McSCs and melanoma, and point to genes whose role in normal and transformed cells could be elucidated in future functional studies.

Considering such interactions raises further questions regarding how transcriptional changes in the McSC lineage impacts other cell types in the surrounding microenvironment, especially HFSCs and their lineage. Numerous studies have described mechanisms through which HFSCs and their HF lineage cells dictate the behavior of McSCs and melanocytes, but whether the reverse scenario is true (outside of providing UV protection) is not well understood. Given that hair growth is not dependent on functional McSCs or their progeny (white hair can grow), the implications of melanocytic signals might be subtle, but perhaps still present. These scRNA-seq
data provide a list of candidate factors that could serve as a starting point for such investigations. Conversely, it still remains to be determined which components of the bulge and HF niche are triggering these gene expression changes in the McSC lineage, as opposed to those that are intrinsically controlled.

Finally, in their cyclic quiescence, activation, transit amplification, and differentiation, McSCs mirror the developmental trajectory of HFSCs. Hsu et al. (2014) demonstrated that HF matrix TACs signal back to quiescent SCs via Sonic hedgehog (SHH) signaling to induce their self-renewal. This begs an intriguing question: do McSC TAC progeny (McCP) provide feedback to their parent SCs in the way that HFSCs do? Based on the timing of this signaling for matrix TACs, the data obtained from aMcSCs and early McCPs in anagen I-II might present clues. Indeed, to create banded patterns in feathers, it is believed that mature melanocytes engage in a negative feedback loop, signaling back to undifferentiated proliferative progenitor cells to stop their progression and achieve cyclic maturation and pigmentation (Inaba and Chuong, 2020).

In summary, these new data contribute a more complete and high resolution picture of McSC transcription throughout differentiation. Notably, this dataset affords a high depth of coverage to discern maximal differences between cell types, and it includes intermediate stages of lineage progression, namely aMcSCs and McCP. Recently, Smart-Seq2 data has been generated for human melanocytes isolated from different patient ages and body sites (Belote et al., 2020). Future cross-species analyses will likely provide useful new insights for the field’s understanding of this lineage.

However, transcription is just one level of regulation impacting cellular dynamics; global proteomic and epigenetic studies at the single cell level will provide further insight into how these different McSC states are achieved and how chromatin state, transcription, and translation
intersect. This work would inform questions concerning whether some qMcSCs, while appearing transcriptionally homogeneous, are actually poised for activation, while another pool is more deeply quiescent. These differential gene expression and pathway analyses implicated both known and novel genes and biological processes at each stage, allowing me to form more specific hypotheses about how McSCs pass through activation and differentiation, which is the subject of Chapter 3 and centers on BMP and WNT signaling (Chapter 2.2.6). At the same time, this body of work raises several diverse hypotheses, and will hopefully form the foundation for future studies in the McSC lineage during homeostasis and in disease.
CHAPTER 3:
BMP SIGNALING DRIVES FULL DIFFERENTIATION OF MELANOCYTES
DOWNSTREAM OF STEM CELL ACTIVATION AND LINEAGE COMMITMENT
3.1 Introduction

By performing high sensitivity scRNA-seq on McSC lineage cells at multiple developmental time points, I gained temporal insight on not only the gene expression changes during lineage progression, but also when signaling pathways seem to become activated. In narrowing the focus of my project to study the pathways and downstream TFs that were likely to impact lineage progression and differentiation, both BMP and WNT signaling featured prominently (Chapter 2.2.6). When compared to the most highly differentiated melanocytes in the dataset (C3c), the later McCP of the anagen VI hair bulb (C3a) showed significant upregulation of genes involved in both pathways. This was intriguing given the essential role of WNT signaling in McSC activation and downstream melanocyte maturation (Rabbani et al., 2011), and also given reports of both cooperative and antagonistic effects of WNT and BMP pathways in SC biology.

Simply described, canonical WNT signaling occurs through the binding of WNT ligands to frizzled (FZD) and lipoprotein receptor-related protein (LRP) receptors, disrupting the cytoplasmic β-catenin degradation complex. Stabilized β-catenin can then translocate to the nucleus and bind with TCF/LEF family TFs to regulate gene expression (Nusse and Clevers, 2017). WNT plays an essential role in the melanocyte lineage, from embryonic specification of melanoblasts, where it appears to act antagonistically with BMP signaling (Hari et al., 2002; Hari et al., 2012; Jin et al., 2001), to activation of HF McSCs and downstream proliferation and differentiation of melanocytes in the hair bulb (Rabbani et al., 2011).

BMP signaling occurs through the binding of BMP ligands to complexes of BMP type I and type II cell surface receptors, triggering their serine/threonine kinase-mediated phosphorylation of SMAD1/5/9 proteins. Phosphorylated SMAD1/5/9 then bind SMAD4 and translocate to the nucleus to regulate target gene expression (Wang et al., 2014). Through genetic
manipulation of Bmpr1a (ALK3), the Fuchs Lab has shown that this pathway is critical for HF lineage regulation. BMP signaling both maintains HFSCs in quiescence and promotes the differentiation of HF TACs (Genander et al., 2014; Kobi et al., 2007). However, the role of BMP signaling in McSC lineage commitment was not well understood, with most insights resting on cell culture studies (Bilodeau et al., 2001; Jin et al., 2001; Kawakami et al., 2008; Park et al., 2009; 2005; Singh et al., 2012; Yaar et al., 2006; Yang et al., 2014); the few studies in mice made the precise contributions of this pathway difficult to interpret (Han et al., 2012; Sharov et al., 2005).

Given the highly context-dependent roles of this pathway, which stem from promiscuity of ligand binding, differential receptor expression and complex combinations, and expression of positive and negative pathway regulators (Wang et al., 2014), I wanted to gain a deeper understand about how BMP works in the lineage in the native tissue microenvironment. Therefore, in my thesis research, I posed the following hypotheses: 1) BMP (BMPR1a) signaling promotes McSC lineage progression and melanocyte differentiation, and 2) BMP signaling coordinates with WNT signaling in the differentiation process. To test these hypotheses, I harnessed genetic mouse models, IMF and ultrastructural analyses, cell culture, scRNA-seq, and chromatin profiling approaches.

3.2 Results

3.2.1 Cocomitant activity of WNT and BMP signaling in the lineage

At the transcript level, there is evidence of increasing WNT and BMP signaling at the late McCP stage. To confirm the acitivy of these pathways at the protein level, I performed IMF analysis of the skin throughout hair cycling. To assay for WNT activity, I interrogated the TF LEF1. While McSCs throughout cycling lacked LEF1, nuclear signal became robust as cells
transitioned to the McCP/melanocyte stages in anagen (Fig. 3.1 A). While inner bulge HFSCs showed robust BMP signaling through pSMAD1/5/9, neighboring McSCs lacked nuclear signal in both their quiescent and activated states. However, as differentiation progressed, McCP/melanocytes in the hair bulb became strongly pSMAD1/5/9+, and this was maintained throughout anagen (Fig. 3.1 B). Together, these RNA and protein analyses were consistent with the hypothesis that BMP signaling acts during differentiation. Furthermore, the concomitant timing of pSMAD1/5/9 and LEF1 nuclear accumulation in McCP/melanocytes in anagen HFs were in agreement with a degree of convergent signaling. On this basis, I proceeded to employ loss-of-function mouse models to investigate the role of BMP signaling in lineage progression and how it might intersect with WNT.
Figure 3.1 WNT/LEF1 and BMP/SMAD signaling is activated in McCP/melanocytes. **(A)** IMF images showing LEF1 protein expression in the melanocyte lineage throughout hair cycling. Nuclear LEF1 (red) indicated by yellow arrows. **(B)** pSMAD1/5/9 (BMP signaling) is absent in McSCs but becomes nuclear in McCPs/melanocytes (Mcs), indicated by yellow arrows. All scale bars=25µm.
3.2.2 Perturbed WNT signaling in mice results in reduced hair pigmentation

To understand the importance of LEF1 in the lineage, I first revisited Lef1 full body KO mice (Kratochwil et al., 1996; van Genderen et al., 1994). These mice had less pigmented hair compared to their littermates (Fig. 3.2 A). Due to their lack of most HFs, there is abnormal localization of melanocytes in KO skin, including pigmented melanocytes in the dermis and some follicles containing fewer melanocytes. However, in the follicles that did contain DCT+ cells in their anagen HFs, hair bulbs and hairs still appeared less pigmented, suggesting that loss of LEF1 impaired melanocyte differentiation (Fig. 3.2, right).

I then used a second, more specific approach to conditionally ablate β-catenin (Ctnnb1) in the lineage by generating TyrCreER Ctnnb1\textsuperscript{flaxed} R26-YFP\textsuperscript{flaxed} mice (Bosenberg et al., 2006; Brault et al., 2001; Srinivas et al., 2001). This model is similar to that used by Rabbani et al. (2011) to demonstrate that WNT signaling plays key roles in McSC activation and downstream proliferation and differentiation of melanocytes in the hair bulb. Upon tamoxifen treatment, TyrCreER drives expression in both McSCs and their differentiating progeny (Bosenberg et al., 2006). At P21, mice were treated with seven consecutive doses of tamoxifen (Fig. 3.2 B). Consistent with the results of Rabbani et al. (2011), by second telogen, Ctnnb1 conditional KO (cKO) mice had hairs with reduced pigmentation relative to control (Fig. 3.2 C). I then wanted to interrogate the status of BMP signaling in the absence of functional WNT/β-catenin signaling. To analyze the McSC lineage during differentiation, I waxed tamoxifen-treated second telogen mice to induce synchronous hair growth and analyzed tissues one week later in anagen (Fig. 3.2 B). Nuclear pSMAD1/5/9 was still present in Ctnnb1 null McSC progeny in the hair bulb, indicating that BMP signaling is not dependent on WNT signaling (Fig. 3.2 D).
Figure 3.2 WNT mutant mice have reduced pigmentation. (A) Left, phenotype of Lefl KO mice at P13, when hair on the skin surface has emerged in KO (control littermate: female, Lefl KO: male). Middle, IMF for LEF1 in control and Lefl KO skin at P0. Right, brightfield images of P8 anagen HFs. (B) Experimental schematic for conditionally ablating β-catenin (WNT) in the melanocyte lineage in vivo. (C) Phenotype of tamoxifen-treated TyrCreER Ctnnb1 floxed R26YFP floxed mice at 2nd telogen. (D) IMF of Ctnnb1 cKO and control skin 1 week after waxing. BMP signaling through nuclear pSMAD1/5/9 is indicated by yellow arrows. Scale bars=25µm.
3.2.3 Loss of BMP signaling through BMPR1a leads to hair graying

To interrogate the role of BMP signaling in McSC lineage progression, I generated a strain of conditional lineage-specific Bmpr1a KO mice, TyrCreER Bmpr1a$^{lox}$ R26YFP$^{lox}$ (Bosenberg et al., 2006; Mishina et al., 2002; Srinivas et al., 2001). BMPR1a is a type I receptor serine/threonine kinase whose loss extinguishes downstream signaling through the pathway. I treated these mice with tamoxifen at P21 and allowed them to progress to second telogen. Strikingly, by this point, Bmpr1a cKOs exhibited gray hair, while Bmpr1a heterozygotes (TyrCreER$^{+}$ Bmpr1a$^{fl/+}$) showed no phenotype relative to controls (TyrCreER$^{-}$ or TyrCreER$^{+}$ Bmpr1a$^{+/+}$) (Fig. 3.3 A). To observe the McCP/melanocyte populations in hair bulb compartment, tamoxifen-treated second telogen mice were waxed, and anagen skin was collected after one week of hair growth. As measured by optical density, there was significantly reduced pigmentation in Bmpr1a cKO hair bulbs (Fig. 3.3 A-B).

Gray hair phenotypes can occur due to defects in the McSC compartment, including apoptosis, ectopic proliferation and differentiation, or migration out of the niche (Nishimura et al., 2011). Alternatively, abnormalities may occur downstream at other points in differentiation (Fig. 1.2). To ascertain the cause of the gray hair in Bmpr1a cKO mice, I waxed second telogen mice to trigger hair growth and McSC activation and differentiation, then analyzed McSCs after one week using IMF. McSCs numbers per HF were comparable between Bmpr1a cKO mice and controls (Fig. 3.3 C) and seemed to return to quiescence normally after activation, as measured by EdU incorporation during a 4 hour pulse (Fig. 3.3 D). To more conclusively rule out McSC dysfunction, I challenged the SC pool by performing serial waxing. The anterior back skin of second telogen mice was waxed five times in total every three weeks, by which time the waxed region appeared to return to telogen. By four rounds of waxing, the anterior hair coat did not show an appreciable
Figure 3.3 Conditional ablation of Bmpr1a in the lineage results in gray hair without compromised McSCs. (A) Representative phenotype of TyrCreER Bmpr1a<sup>floxed</sup> R26YFP<sup>floxed</sup> mice tamoxifen-treated mice in 2nd telogen. Scale bars=25µm. (B) Quantification of pigment content in hair bulbs measured by optical density. ****p<0.0001, Mann-Whitney test; Control n=80 HFs (n=4 mice); Bmpr1a cKO n=43 HFs, (n=3 mice). (C) Quantification of McSCs numbers per anagen HF 1 week after waxing [*p=0.0184, Mann-Whitney test; Control n=76 HFs (n=5 mice); Bmpr1a cKO n=45 HFs (n=4 mice)]. (D) Quantification of EdU<sup>+</sup> McSCs per HF 1 week after waxing [(ns=not significant, Mann-Whitney test; Control n=61 HFs (n=3 mice); Bmpr1a cKO n=45 HFs (n=4 mice)] (E) Phenotype of control and Bmpr1a cKO mice after 4 rounds of waxing on the anterior portion of the back skin, 3 weeks apart. Note that a small skin biopsy was taken from the posterior midline near the time of initial waxing of the anterior. (F) Quantification of McSCs per HF after 5 rounds of depilation, quantified in anagen 1 week after the fifth wax [(Mann-Whitney test; Control n=30 HFs (n=3 mice); Bmpr1a cKO n=40 HFs (n=4 mice)].
exacerbation of hair graying (Fig. 3.3 E). Anagen skin was collected one week after the fifth wax for IMF, and I found no significant difference in number of McSCs per HF between control and Bmpr1a cKO mice (Fig. 3.3 F). These results suggested that McSCs were intact and functional upon Bmpr1a loss and were consistent with my scRNA-seq (Fig. 2.7) and IMF data (Fig. 3.1 B), which both indicated that this pathway is not activated until the McCP stage.

I therefore turned my attention to identifying defects downstream in the lineage. In Bmpr1a cKO mice, DCT+ cells populated the McCP/melanocyte compartment of the hair bulb. By IMF, these cells showed loss of nuclear pSMAD1/5/9 as well as pP38, indicating ablation of canonical and non-canonical pathways downstream of BMPR1a, respectively (Fig. 3.4 A-B). To rule out the possibility that hair bulbs were less pigmented because cells were simply delayed in differentiation, I analyzed skin further into anagen. Indeed, 12 days after waxing, Bmpr1a cKO hair bulbs were still poorly pigmented (Fig. 3.4 C). These data suggested that without BMP signaling, McSC quiescence and activation were not impacted, but lineage progression and subsequent melanin production were disrupted.
Figure 3.4 McSCs in TyrCreER\(^{+}\) Bmpr1a\(^{+/\beta}\) R26YFP mice give rise to differentiating progeny that populate the hair bulb. (A) IMF showing loss of BMP-SMAD signaling through pSMAD1/5/9 and (B) MAPK through pP38 in Bmpr1a null DCT\(^{+}\) cells in the hair bulb. (C) Bright field images of control and Bmpr1a cKO hair bulbs 12 days after waxing. Scale bars=25\(\mu\)m

3.3.4 ScRNA-seq and pseudotime analysis of Bmpr1a null cells in vivo suggests a block in differentiation between early McCP and mature melanocyte

Given that my in vivo results suggested a defect downstream in differentiation, I sought to gain further mechanistic insights using a cultured melanocyte mouse cell line, Melan-a2 (Sviderskaya, et al., 1995). To minimize variability, I first generated a clonal parental line from Melan-a2, then using CRISPR/Cas9, I generated and validated Bmpr1a KO and control clonal cell lines (Fig. 3.5 A). However, while loss of the receptor in culture led to reduced pSMAD1/5/9 upon BMP2 stimulation, signaling was not completely abolished, and BMP6-induced pSMAD1/5/9 signaling was not affected in Bmpr1a KO cells (Fig. 3.5 B). At the same time, no apparent change in melanin was observed, suggesting that the in vivo phenotype could not be
Figure 3.5 FACS purification of in vivo Bmpr1a null cells preserves the molecular profile that results from the dynamic and complex microenvironment. (A) Validation of CRISPR KO of Bmpr1a using Cas-analyzer tool on rgenome.net, using MiSeq results. (B) Western blot analysis of pSMAD1/5/9 signaling in control and KO Melan-a2 cell lines after 1 hour BMP stimulation (0.5% serum, CT-free media during treatment). (C) IMF images of skin at different stages of hair cycling. Bu=bulge, HB=hair bulb. Scale bars=50µm. (D) FACS purification schemes for YFP+ populations. (E) Quality control metrics showing log number of counts, genes/cell, and percentage of mitochondrial genes for unfiltered datasets. (F) Unsupervised k-NN based clustering of control (heterozygous, Het and wild-type, WT) and Bmpr1a null cells colored by known identity (left) and individual mouse (“M”) (right). For cell identity, purple are WT, (TyrCreER+ Bmpr1a+/+, n=205 cells, n=1 mouse), blue are Het, (TyrCreER+ Bmpr1a+/-, n=304 cells, n=2 mice) and green are Bmpr1a null (TyrCreER+ Bmpr1a+/-, n=421 cells, n=2 mice).

accurately recapitulated in vitro. This was not surprising given the complexity and context-dependent behavior of this pathway. BMP/TGF-β receptor superfamily members (i.e. activin receptors) can dimerize in multiple combinations and yield signaling responses that differ depending upon the specific ligand, receptors, and intra- and extracellular regulator molecules present (Wang et al., 2014). These differences limited the studies I could perform in culture, but
provided a plausible explanation for the context-dependent behaviors described in this lineage in the literature (Bilodeau et al., 2001; Han et al., 2012; Jin et al., 2001; Kawakami et al., 2008; Park et al., 2009; Sharov et al., 2005; Singh et al., 2012; Yaar et al., 2006; Yang et al., 2014). These findings underscored the importance of taking *in vivo* approach where possible to study this signaling pathway and specific receptor’s function.

Therefore, I focused on methods that enabled me to study the effect of BMPR1a loss in mice. To this end, I turned again to FACS purification and scRNA-seq. *Bmpr1a* control (*Bmpr1a* heterozygous +/fl and WT +/+) and cKO mice were treated with tamoxifen at P21, allowed to progress to second telogen, then waxed to induce anagen. At one week post-waxing, HFs were in anagen IV, an ideal time point at which to capture differentiating McCP in the hair bulb when BMP signaling becomes activated (Fig. 3.5 C). Using a digestion protocol to preferentially release cells from the dermis of the skin including hair bulb cells, I FACS purified YFP+ cells, gating against CD31, CD45, CD140a, Sca-1 and CD34+ cells (Fig. 3.5 D) and again performed scRNA-seq. Again, this approach afforded many advantages, including the ability to discern any YFP+ cells captured that were not actually *Bmpr1a* null (which were consequently clustered appropriately with control cells). ScRNA-seq data were subject to quality control analyses and filtering parameters, where any *Dct*low and *Krt15*+ cells were again excluded from further analysis. Overall, cells displayed a similar depth of sequencing with approximately 5,000 genes per cell (consistent with increased transcriptional activity occurring during differentiation), and few reads mapped to mitochondrial transcripts (Fig. 3.5 E). Furthermore, independent replicates exhibited similar clustering (Fig. 3.5 F).

PCA with UMAP and k-NN clustering of these data revealed the presence of two major groups. *Bmpr1a* WT and heterozygous cells showed significant overlap and comprised one of
these two clusters, while \textit{Bmprla} null cells formed the second cohort, indicating their unique transcriptome (Fig. 3.6 A). Consistently, expression of direct BMP target genes \textit{Id2} and \textit{Id3} were dramatically downregulated in this \textit{Bmprla} null cluster (Fig. 3.6 B). To understand what other transcriptional differences were driving this separation, differential gene expression analysis was performed on the control versus \textit{Bmprla} null cluster. This indicated that 80 genes were significantly (q\geq0.05) downregulated and 346 genes were upregulated in the absence of \textit{Bmprla} (Fig. 3.6 C-D; Table 3.1-3.2; see also Table S9 of Infarinato et al., 2020). Strikingly, \textit{Bmprla} null cells showed diminished expression of genes involved in melanogenesis and melanosome organization, including \textit{Oca2, Rab38, Gpr143, Shroom2,} and \textit{Mc1r}. At the same time, these cells
Figure 3.6 *Bmpr1a* null cells downregulate melanogenesis, melanosome, and transport genes. (A) UMAP representation and unsupervised k-NN based clustering of control and *Bmpr1a* null cells colored by cluster identity. (B) Expression plots of *Id2* and *Id3* in YFP+ populations. (C) Heatmap of top differentially expressed genes for *Bmpr1a* null versus stage-matched controls and (D) selected GO terms (DAVID 6.8) with (p-values). See also Table 3.1-3.2. (E) RT-qPCR expression of genes from scRNA-seq list isolated 12 days post waxing ([* ****p<0.0001 Bmpr1a; ** p=0.0035 Lef1; * p=0.0269 Eps8; * p=0.0148 Vimentin; *** p=0.0010 Pax3, Unpaired t-test; Heterozygous control n=3 mice, Bmpr1a cKO n=4 mice]).
showed elevated transcripts of genes involved in stemness (*Pax3, Vim*), migration and cellular projections (*Enpp2, Tubb2b*), and cell adhesion (*Cspg4, Adgrg1*). To test whether these trends persisted further into anagen, bulk YFP<sup>+</sup> cells were FACS purified 12 days after waxing for RT-qPCR, which produced similar results for several genes (**Fig. 3.6 E**).

These intriguing results begged another question: if integrated into the original WT McSC lineage dataset (**Chapter 2**), how would the *Bmpr1a* null cells compare? Pseudotime algorithms can be implemented on scRNA-seq data to gain temporal information about a biological process such as SC differentiation, ordering individual cells in a dataset along an inferred trajectory. Therefore, these experimental datasets were integrated with the six WT lineage sub-clusters (qMcSCs, aMcSCs, early and late McCP, and differentiated melanocytes) and UMAP-based dimensionality reduction and clustering was performed (**Fig. 3.7 A**). One-third of *Bmpr1a* null cells clustered with early McCP (C2b), suggesting their transcriptional similarity (**Fig. 3.7 B, left**).

Next, the collective dataset was subject to pseudotime analysis using Monocle3 (Cao et al., 2019), a relatively unbiased method to map differentiation in which all biologically significant genes are considered. As expected based on the hair cycle time point from which cells were isolated and the differential gene expression analyses between sub-clusters (**Fig. 3.7 B**), qMcSCs (C1) were placed at the start or “root” of the trajectory, followed by aMcSCs, then McCP, with the most differentiated melanocytes (C3c) forming the end point of the “branch.” As expected, Monocle3 also indicated that *Bmpr1a* control cells collected from anagen IV hair bulbs were earlier in the differentiation process than all WT McCP/melanocyte (C3) sub-cluster cells isolated from anagen VI hair bulbs. *Bmpr1a* null cells however were placed after early McCP (C2b) from anagen I-II HFs and their stage-matched controls, as well as all other anagen VI McCP/melanocytes (C3).
Their placement in the pseudotime map suggested that Bmpr1a null cells were blocked in their differentiation trajectory. To further explore this idea, the expression of established “stemness” and “differentiation” signature genes (Fig. 2.5 B) was analyzed relative to the WT lineage sub-clusters (Fig. 3.7 C). Bmpr1a null cells showed reduced expression of genes involved in melanogenesis as compared to the WT McCP/melanocytes (C3), as well as genes involved in BMP and WNT signaling pathways, with elevated expression of stemness genes. This is visualized through the global scoring system in the expression plots in Fig. 3.7 D, where levels of stemness and differentiation gene scores can also be compared to a small number of YFP+ McSCs collected from both Bmpr1a control and null groups, which have the highest stemness and lowest differentiation scores. Overall, these analyses suggested that Bmpr1a null cells share many transcriptional similarities with early McCP but have elevated melanogenesis and differentiation-associated gene expression, underscoring that these cells are arrested just downstream of the aMcSCs and early McCP stages.
Figure 3.7 Pseudotime analysis of the Bmpr1a null cells suggests a block at the juncture after early McCP. (A) Unsupervised k-NN based clustering of original melanocyte lineage populations with Bmpr1a null and control cells, colored by sub-cluster identity. (B) Pseudotime analysis of combined datasets colored by pseudotime clusters (left) and pseudotime order (right). Cells are colored by progression through pseudotime with dashed red arrow to indicate direction of progression. (C) Heatmap showing expression of key signature genes in Bmpr1a null cells relative to WT sub-clusters. (D) Expression plots of global stemness and differentiation scores for Bmpr1a null and control cells.

3.3.5. Bmpr1a null cells in the hair bulb exhibit signs of immaturity

My transcriptional data suggested that Bmpr1a null lineage cells were blocked in differentiation. Therefore, I performed tissue analyses to test for features of melanocyte immaturity. To test for proliferation, I treated mice with tamoxifen at P21, waxed their backskin in second telogen, and collected anagen skin one week later after a 4 hour EdU pulse. In agreement
with their transcriptional similarities to aMcSCs and early McCP, Bmpr1a null cells in the hair bulb showed increased EdU incorporation relative to control (Fig. 3.8 A). I confirmed these results in another experiment, where I counted DCT+ cells in the hair bulb and found cell numbers to be significantly increased in Bmpr1a cKOs (Fig. 3.8 B). This suggested that surprisingly, the reduced pigmentation in the cKO hair bulbs was not due to reduced numbers of McCP/melanocytes. This contrasted from the effect of loss of WNT/β-catenin signaling, which results in reduced proliferation in both the McSC and hair bulb compartments (Rabbani et al., 2011). At the same time, Bmpr1a null cells in the hair bulb exhibited increased expression of the McSC signature gene vimentin at the protein level, further indicating their immaturity (Fig. 3.8 C).

Figure 3.8 Bmpr1a null cells in the anagen hair bulb exhibit signs of immaturity. (A) 4 hour EdU pulse experiment with representative IMF images and quantification [****p<0.0001, Mann-Whitney test; control n=50 HFs (n=2 mice); Bmpr1a cKO n=75 HFs (n=3 mice)]. Yellow arrow heads indicate proliferating (EdU+) DCT+ cells. (B) Quantification of DCT+ cells per hair bulb 1 week after waxing [****p<0.0001, *p=0.0103, Kruskal-Wallis test, Dunn’s multiple comparisons test; TyrCreER- control n=70 HFs (n=3 mice); Het control n=63 HFs (n=3 mice); Bmpr1a cKO n=62 HFs (n=4 mice)]. (C) IMF expression of vimentin (red); yellow dashed area encircles DCT+ cell area. Scale bars=25µm

Given the presence and increased number of McSC differentiating progeny in Bmpr1a cKO hair bulbs, I next tested for the protein expression of the three key melanogenic enzymes: DCT, tyrosinase (TYR), and tyrosinase-related protein 1 (TYRP1). In agreement with my scRNA-seq
data indicating that these enzymes are upregulated earlier on in differentiation, DCT, TYR, and TYRP1 (which was in fact reduced in Bmpr1a null cells at the transcript level) were all present by IMF in Bmpr1a null cells in the hair bulb (Fig. 3.9 A-B), suggesting that the defect in pigmentation was unlikely attributable to loss of any one critical component of melanin production. Instead, I turned to assessing melanosomes. While PMEL, a structural component of melanosomes involved in organelle maturation, was expressed in Bmpr1a null cells, it exhibited a more perinuclear localization compared to control cells, where labeling was seen throughout the cell body and dendritic processes (Fig. 3.9 B, right). This suggested a perturbation in melanosome biogenesis or localization, which was reinforced by the reduced expression of melanosome organization genes seen by scRNA-seq (Fig. 3.6 C-D).

Melanosome biogenesis proceeds through four distinct phases of maturation that are well-defined at the ultrastructural level. Stage I melanosomes largely resemble endosomal multivesicular bodies, stage II melanosomes exhibit parallel intraluminal striations, stage III melanosomes show thickening and darkening of these striations as melanin is deposited, and stage IV mature melanosomes ultimately become opaque (Marks and Seabra, 2001). To investigate the status of melanosomes in Bmpr1a null cells, back skin was waxed and collected one week later in anagen for electron microscopy (EM) of lineage cells in the hair bulb. Quantification of total and mature (stage IV) melanosome area densities revealed similarities between control and Bmpr1a null cells, but Bmpr1a null cells displayed a significantly higher density of immature (stage I-III) melanosomes (Fig. 3.9 C). Together, these results suggest that in the absence of BMPR1a signaling, McSC progeny are suspended in a partially differentiated state characterized by increased proliferation and perturbed melanosome biogenesis and pigment production.
Figure 3.9 *Bmpr1a* null cells are intermediately differentiated. (A) IMF images showing expression of early melanogenic enzymes in control and *Bmpr1a* null cells in the hair bulb. (B) IMF image showing expression pattern of PMEL. Area within yellow dashed box is enlarged to the right, with yellow arrows to indicate perinuclear localization of signal. All IMF scale bars=25µm. (C) EM images of silver sections with melanocyte lineage cells outlined in green. Dashed box insets are enlarged. Red arrows indicate immature melanosomes. Scale bar=1µm. Lower graphs show quantification of number of melanosomes divided by melanocyte lineage cell area. Left – total melanosomes/µm², middle – mature melanosomes/µm², right – immature melanosomes/µm² [*****p<0.0001, Mann-Whitney test; Control n=66 areas (3 mice); Bmpr1a cKO n=52 areas (3 mice)*].
3.3.6 BMP-regulated *Lef1* may cooperate with MITF to drive full melanocyte differentiation

Given the newfound role of BMPR1a signaling at the juncture downstream of McSC activation/early McCP and upstream of melanocyte differentiation (Fig. 3.10 A), I sought further insight into how this signaling cascade is transmitted to regulate genes involved in differentiation and melanosome organization. My overarching hypothesis for my thesis work was that distinct transcriptional programs are fueled by specific TFs that drive dynamic cellular states throughout differentiation. Therefore, I turned my attention to TFs that were downregulated in Bmpr1a null cells by scRNA-seq. Intriguingly, WNT mediator *Lef1* showed a trending downregulation by scRNA-seq (Fig. 3.10 B, left) and RT-qPCR (Fig. 3.6 E). Consistently, Bmpr1a null cells exhibited reduced nuclear LEF1 levels by IMF (Fig. 3.10 B, right).

Probing for further changes in TFs, I interrogated MITF, the master regulator of the melanocyte lineage that is often regulated at the post-transcriptional level (Goding and Arnheiter, 2019). MITF coordinates myriad lineage processes in development, differentiation, and melanoma, including survival, proliferation, and pigment production (Goding and Arnheiter, 2019). Carreira et al. (2006) proposed that in melanoma, MITF functions according to a “rheostat model,” where low MITF promotes stemness, intermediate MITF induces proliferation, and high MITF drives differentiation and melanogenesis. While *Mitf* levels were unchanged at the transcript level in Bmpr1a null cells relative to control, interestingly, nuclear MITF protein was reduced by IMF (Fig. 3.10 C).

Given the numerous differentiation and pigmentation defects that stem from perturbations or loss of MITF, reduced nuclear MITF in Bmpr1a null cells alone might largely explain the block in melanocyte differentiation and resulting gray hair phenotype. However, MITF is known to regulate its diverse cellular processes through cooperation with other TFs (Goding and Arnheiter,
Figure 3.10 Nuclear LEF1 and MITF are reduced in differentiating Bmpr1a null cells. (A) Model of McSC lineage progression with the proposed timing of the differentiation block without BMP signaling. (B) *Lef1* mRNA expression (left) with IMF (right) in control and *Bmpr1a* null cells. Yellow arrows indicate presence and white arrows indicate reduction/absence of nuclear LEF1. (C) IMF images depicting MITF in control and *Bmpr1a* null cells in anagen 1 week after waxing. Enlarged areas show reduction (white arrows) of nuclear MITF in transduced (YFP+) vs. un-transduced (YFP-) as an internal control. (D-E) IMF images illustrating MITF levels in hair bulb McSC progeny of (D) control and *Lef1* KO (P4) mice and (E) *Ctnnb1* cKO and control mice whose HF s were analyzed 1 week post-waxing. Yellow arrows indicate presence and white arrows indicate reduction/absence of nuclear MITF. IMF scale bars=25µm.

Therefore, given that *Bmpr1a* null cells also showed reduced LEF1, I wanted to investigate the link between BMPR1a signaling, WNT/LEF1, and MITF. Moreover, previous reports have indicated that LEF1 can physically interact with and regulate MITF (Dorsky et al., 2000; Saito et al., 2002, Takeda et al., 2000; Yasumoto et al., 2002).

To ask whether such an interaction might be occurring, I first examined McSC progeny in the hair bulbs of *Lef1* KO and *Ctnnb1* cKO mice and also found that these cells exhibited
Figure 3.11 BMP-stimulated cells upregulate Lef1/LEF1. (A) Id2 and Lef1 mRNA levels by RT-qPCR after 30 hours of stimulation with 10 ng/mL recombinant BMP2 and BMP4 ± 250 nM ALK inhibitor (ALKi) [(BMP2 graphs: Id2, ****padj=0.0001, **padj=0.0084; Lef1, ***padj=0.0001) (BMP4 graphs: Id2, ****padj=0.0001, Lef1, ****padj=0.0001, *=0.0111 by ordinary one-way ANOVA, Dunnett’s multiple comparisons test)]. (B) pSMAD1/5/9 and LEF1 protein levels by western blot after 48 hour treatment. (C) Expression plot of Mitf levels in control and Bmpr1a null cells. (D) Mitf levels by RT-qPCR after 30 hours of stimulation with 10 ng/mL recombinant BMP2 ± 250 nM ALKi, with MITF protein levels by western blot after 48 hour treatment.

diminished nuclear MITF (Fig. 3.10 D-E), further suggesting a link between these signaling pathways and TFs. While cell culture experiments were limiting overall (see Chapter 3.3.4.), I did find that upon BMP2/4 stimulation (ligands that are expressed in anagen skin in vivo), Lef1 was upregulated. LEF1 protein levels were also increased at 48 hours of treatment with BMP2, as assessed by western blot. These trends were reversible upon treatment with the BMP receptor
(ALK) inhibitor K02288 (Fig. 3.11 A-B). While Mitf mRNA was not changed during differentiation (Fig. 3.11 C) or upon BMP stimulation in culture (Fig 3.11 D, left), in vivo, there was increased MITF protein upon intact BMPR1a signaling (Fig. 3.10 C). However, in Melan-a2 cells, MITF levels were already high in untreated cells and did not change further upon BMP2 stimulation or K02288 inhibitor treatment (Fig. 3.11 D, right), again underscoring the importance of studying this signaling cascade in the lineage in the physiological context.

To further investigate the possibility of such pathway intersection, genes with reduced expression in Bmpr1a null cells relative to control by scRNA-seq were scanned for TF motifs within their putative promoter regions (within 2.5 kb upstream of the transcription start site, TSS). To assess interplay between MITF and BMP-regulated LEF1 TFs, genes were classified as containing a MITF motif only, a LEF1 motif only, or both. In another analyses, to assess MITF and BMP-mediated pSMAD1/5/9 cooperation, genes were binned as containing a MITF motif only, a SMAD motif only, or both. These analyses revealed that genes with promotors harboring both MITF and LEF1 sites showed significantly lower expression than those with MITF alone; this preferential downregulation was not seen in genes with MITF and SMAD relative to MITF alone (Fig. 3.12 A). Analyses of genes increased in Bmpr1a null cells did not indicate any enrichments (Fig. 3.12 B). Strikingly, the BMP-regulated genes with both MITF and LEF1 sites (23 genes total) included those specifically involved in transport, transmembrane transport, melanin production, and melanosome organization, including Mc1r, Oca2, Trpm1, Rab38, and Shroom2 (Table 3.3; see also Table S11 of Infarinato et al., 2020). Together, these results suggested that downstream of BMPR1a signaling, LEF1 and MITF accumulate in the nucleus, where they may cooperate in the upregulation of differentiation-associated genes, particularly those involved in melanosome organization and transport.
3.3.7 Chromatin accessibility dynamics during the transition from McSC to melanocyte suggests cooperation between LEF1 and MITF in differentiation

To look for further evidence of that MITF and LEF1 cooperate in melanocyte differentiation in vivo, I lastly sought to interrogate the chromatin landscape of the lineage. Recent studies have indicated the importance of specific chromatin modifiers in regulating McSC behavior (Koludrovic et al., 2015; Moon et al., 2017), but again, I wanted to take an unbiased approach to survey chromatin in vivo. I hypothesized that chromatin openness would change as McSCs underwent differentiation into melanocytes and that regions that gain accessibility during differentiation would contain TF binding motifs that play a role in this transition. To assay chromatin dynamics in the McSC lineage, I implemented Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq), a technique that uses Tn5 transposase
Figure 3.13 qMcSCs and their progeny exhibit differential chromatin accessibility. (A) Correlation matrix indicating similarity between biological replicates of ATAC-seq. (B) Location of ATAC peaks expressed as percentages. (C) Heatmap of differential peaks in qMcSCs versus their differentiating hair bulb progeny (prog.) (Log$_2$FoldChange$\geq$1, p$\leq$0.05).

To insert sequencing adaptors in regions of open chromatin that is suitable for low cell number input ($\geq$10,000 cells) (Buenrostro et al., 2013). For this assay, I isolated qMcSCs from the telogen bulge and McCP/melanocyte progeny from the anagen VI hair bulbs of Dct-eGFP$^+$ mice to evaluate and compare chromatin states in stem and differentiating states.
These ATAC-seq data showed high reproducibility across biological replicates (Fig. 3.13 A), with similar percentages of peaks within 50 kilobases (kbs) of canonical TSSs (28.2% for peaks increased by log$_2$fold≥1 in qMcSCs; 26.4% for McSC progeny) and similarly distributed throughout promoters, gene bodies, and intergenic regions (Fig. 3.13 B). To ask whether the chromatin landscape changes throughout differentiation, differential peak analysis was performed. This analysis indicated that indeed qMcSCs and their differentiating progeny exhibit strikingly distinct chromatin accessibility profiles (Fig. 3.13C [log$_2$ fold change≥1, p≤0.05], see also Table S12 of Infarinato et al., 2020), allowing for examination of chromatin regions containing BMP-sensitive genes by scRNA-seq as well as TF motifs within these dynamic regions.

Genes whose expression was diminished in Bmpr1a null cells showed increased accessibility in differentiation. Example gene tracks for Gpr143 and Oca2 are shown in Fig. 3.14 A. Notably, Lef1 seemed to show chromatin remodeling as McSCs became differentiated, and some of the peaks that became more open in differentiating progeny contained SMAD motifs (Fig. 3.14 B), supporting the notion that this TF gene might be a direct BMP target gene. Consistent with the timing of BMP signaling activity in the lineage, a number of these differential peaks associated with differentiation resided within the promoter/enhancer regions of genes downregulated upon Bmpr1a ablation (Fig. 3.14 C).

To next ask what TFs might bind in these differentially accessible chromatin regions, unbiased TF motif analysis was performed for stem and differentiation-enhanced ATAC peaks. Strikingly, LEF/TCF and MITF (TFEB, TFE3, TFEC) family factors were the top most enriched factors in peaks increased in differentiation (Fig. 3.15 A; see also Table S13 of Infarinato et al., 2020). Conversely, peaks increased in qMcSCs showed enrichment of SP/KLF and AP-1 family motifs, consistent with the enrichment of these factors by RNA-seq, as well as those expected to
Figure 3.14 BMP-sensitive genes show increased accessibility in differentiation. (A) ATAC tracks showing differential peaks of select differentiation genes. (B) ATAC track of Lef1 gene showing chromatin opening in differentiating progeny in regions with SMAD motifs, indicated by dashed red boxes. Merged (mean averaged) of three biological replicates are shown. Zoomed in views shown to bottom left within red dashed lines. (C) Number of peaks increased in either qMcSCs or their differentiating progeny within 5 or 50kb ± of the TSS of genes (80) whose expression is diminished upon loss of BMPR1a signaling by scRNA-seq.

play a role in SC biology, such as PAX3 and RBPJ. Motifs such as SOX10 were present in both qMcSC and differentiating progeny peaks, with no significant enrichment in either direction. Honing in specifically on the set of genes whose expression was diminished in Bmpr1a null cells, another motif analysis was performed on differentiation-enriched ATAC peaks within ±50 kb of the TSS for these differentially expressed genes (putative promoter and enhancer regions). Again, there was significant enrichment of binding motifs for LEF/TCF and MITF family factors (Fig.
3.15 B; Table 3.4; see also Table S14 of Infarinato et al., 2020), further highlighting the importance of both LEF1 and MITF downstream of BMPR1a signaling in driving differentiation.

Lastly, to assess how BMP regulation via SMADs might impact chromatin dynamics, all differentiating progeny peaks were considered for genes whose expression was reduced in Bmpr1a null cells, as analysis of differentiation-enhanced peaks did not yield clear results for SMAD factors. In agreement with the predictive motif scanning of putative TSS regions (Fig. 3.12), analysis of ATAC peaks in the differentiating progeny suggested that many genes involved in melanin biosynthesis, transport, and trafficking (i.e. Mc1r, Rab38, Trpm1, Slc24a4) may require cooperation of SMAD as well as MITF and LEF1 in controlling their lineage-specific activation (Fig. 3.15 C; see also Table S15 of Infarinato et al., 2020). Overall, these data suggest that McSCs and their differentiating McCP/melanocyte progeny undergo chromatin rearrangement during differentiation and provide compelling evidence for not only the master regulator MITF but also LEF1 in promoting lineage maturation.
Figure 3.15 Motif analyses within ATAC peaks suggests cooperation of MITF, LEF1, and SMAD TFs at BMP-sensitive genes. (A) Plot expressing enrichment of select motifs in differential ATAC peaks between each cell type. Motifs with $-\log_{10}(p\text{-value})$ greater than 300 are expressed as a maximum of 300 here. (B) Motif analysis within peaks subset by the following parameters: peaks enriched in differentiating progeny relative to McSCs and 50 kb ± TSS of genes downregulated in Bmpr1a null cells. X. *=MITF family, **=LEF/TCF family. See also Table 3.4. (C) Motif analysis within differentiating McSC progeny peaks within 50 kb ± TSS of genes whose expression is diminished in Bmpr1a null cells. Table highlights LEF1, MITF, and SMAD(2, 3, and/or 4) motifs. Bolded genes specify those encoding proteins involved in melanogenesis.

3.3 Discussion

The temporal nature of my scRNA-seq dataset made it a powerful tool with which to elucidate the precise coordination of niche pathways and TF responses that might interact to achieve aMcSC and McCP proliferation and final melanocyte differentiation. Intrigued by evidence of converging BMP and WNT signaling downstream of McSC activation, I investigated the roles and intersection of these pathways in the lineage harnessing genetic mouse models, IMF and EM analyses, and scRNA and ATAC-seq. These studies revealed a newfound role for signaling through BMPR1a in driving cells past the McCP stage to fully mature melanocytes through nuclear accumulation of MITF and LEF1 to upregulate transcription of melanosome organization and transport genes.
Similar to the results of cell culture studies pertaining to BMP signaling, prior reports in animals were also difficult to resolve. Mice ectopically expressing the secreted BMP inhibitor noggin under the Krt5 epidermal promoter had darker coats due to reduced expression of ASIP, which signals through MC1R to promote pheomelanin production (Sharov et al., 2005). At the same time, mice with melanocyte-specific deletion of Bmpr2 had no phenotype unless on an activin A receptor type 2A (Acvr2a) full body KO background, in which case mice had gray hair (Han et al., 2012). In zebrafish, BMP signaling via growth differentiation factor (GDF6, also known as BMP13) suppresses melanocyte differentiation during development and in melanoma via downregulation of mitfa (an ortholog of Mitf). In implementing a conditional, lineage-specific ablation approach to delete Bmpr1a, a type I BMP receptor with preferential BMP ligand specificity, my approach allowed me to dissect the contribution of this pathway in lineage progression in the mammalian context, allowing for normal melanoblast specification and McSC establishment while preserving the native niche microenvironment. In this way, I could clearly demonstrate the BMP signaling is critical for normal melanocyte maturation, and in contrast to what has been described in zebrafish, promotes nuclear accumulation of MITF, as well as LEF1, to do so.

McSCs must coordinate their behavior with neighboring HFSCs, so because BMP (BMPR1a) signaling maintain HFSC quiescence (Adam et al., 2018; Genander et al., 2014; Kobielak et al., 2007), this pathway might have also been predicted to promote a quiescent state for McSCs in telogen. Surprisingly however, McSCs appear to be largely refractory to BMP signaling, as reflected by the paucity of nuclear pSMAD1/5/9 in these cells throughout the hair cycle. In Bmpr1a cKO mice, McSCs still became activated at early anagen, were able to generate differentiating progeny that populated the hair bulb, and then returned to quiescence later in
anagen. There are several possible scenarios that might explain these distinct responses of McSCs and HFSCs, from differential receptor expression to ligand availability. It is notable that these SCs occupy distinct locales within the niche; quiescent HFSCs flank the BMP-rich inner bulge, while qMcSCs reside near the base of the bulge where BMP levels lower (Hsu et al., 2011; Yang et al., 2017). TGF-β signaling is elevated at the bulge base (Oshimori and Fuchs, 2012), which could explain the preferential sensitivity of McSCs to this related signaling pathway during quiescence (Nishimura et al., 2010). Another interesting possibility is that in the McSC lineage, TGF-β and BMP signaling act in an antagonistic fashion, pushing cells towards stemness/quiescence and differentiation, respectively.

In considering how BMP intersects with WNT, while WNT/β-catenin signaling impacts McSC proliferation (Rabbani et al., 2011), BMP signaling functions after initial McCP specification, when cells are proliferative but show considerable signs of differentiation. In Ctnnb1 cKO mice, nuclear pSMAD1/5/9 was still seen in McSC hair bulb progeny, suggesting that BMP signaling is not dependent on WNT signaling, but nuclear LEF1 was diminished in Bmpr1a null cells, suggesting WNT’s reliance on BMP signaling downstream during melanocyte maturation. Placing BMP signaling upstream of Lef1 expression and nuclear accumulation of LEF1 and MITF provided mechanistic understanding of the detrimental effects of loss of BMPR1a on pigment production, as well as their failure to progress appreciably past the immature McCP stage. While the “rheostat model” alone might explain why some MITF target genes were still expressed in Bmpr1a null cells (i.e. low levels induce expression of genes involved in survival and proliferation) and others are diminished (i.e. high levels induce genes involved in differentiation such as melanosome biogenesis and transport), these data also suggest cooperation with the TF LEF1 to completely achieve differentiation. While Lef1 null lineage cells in the hair bulb showed
reduced nuclear MITF, the mechanism for how these two TFs might be coordinating their behavior it is not yet clear.

While some of BMP’s effect on the lineage appear to be indirect, motif analysis of differentiating melanocyte ATAC peaks provides insight into genes that might be direct SMAD targets, such as *Slc45a2* and *Tyrp1* (SMAD sites only), but further studies are required to demonstrate such regulation. More interesting however was the reduced transcription of genes with both LEF1 and MITF in their putative promoter regions in *Bmpr1a* null cells, and the importance of these factors was reinforced by chromatin accessibility profiling of McSCs versus their differentiating progeny. These ATAC data also revealed that regions within the *Lef1* gene locus with SMAD motifs show enhanced accessibility upon differentiation, corroborating the finding that *Lef1* mRNAs are downregulated upon *Bmpr1a* ablation and suggesting that *Lef1* is likely a direct BMP target gene. Together, these data provide new insights into how BMP signals in this lineage, supporting a model whereby signaling through BMPR1a upregulates *Lef1*, and LEF1 then promotes nuclear accumulation of and cooperates with MITF to govern a critical cohort of melanocyte maturation genes.
CHAPTER 4:
SUMMARY AND PERSPECTIVES
4.1 Summary: new insights into McSC lineage progression and a key role for BMP signaling in melanocyte differentiation in the HF niche

Dissecting the molecular mechanisms that drive adult tissue SC behavior is of fundamental importance for our understanding of how the body maintains and replenishes itself. These same mechanisms can be disrupted in aging and disease. In my thesis research, I sought deeper understanding of McSCs and their dynamic lineage, generating a high-resolution characterization of the transcriptional programs that accompany lineage progression. Importantly, this work has yielded insight into the intermediate stages of McSC activation and McCP maturation and has uncovered that transcriptional heterogeneity increases over the course of the differentiation trajectory (Chapter 2). Using conditional lineage-specific ablation in mice, I have demonstrated a role for BMPR1a signaling in driving complete differentiation, clarifying a role for this pathway in regulating McSC lineage progression in the HF niche that was not well appreciated or understood. Using single cell transcriptional profiling coupled with pseudotime analysis, I have unearthed the complex gene regulation downstream of this receptor and pinpointed where signaling acts in differentiation. Curiously, Bmpr1a null cells retain stemness markers, show elevated proliferation, and have a higher density of immature melanosomes. Importantly, BMPR1a signaling is necessary for the nuclear accumulation of master regulator MITF and WNT mediator LEF1. Investigating the substantial chromatin rearrangements that take place as cells transition from SCs to differentiated melanocytes, motif analyses further pointed to cooperation between MITF and LEF1 near BMP-sensitive genes (Chapter 3). Altogether, my data support a model wherein McSCs are refractory to BMP until the McCP stage, when signaling promotes nuclear accumulation of MITF and SMAD-mediated upregulation of Lef1/LEF1; these two key TFs regulation transcription of genes required for terminal differentiation, involving the upregulation of melanosome and transport genes and the downregulation of stemness and proliferation genes.
In considering how these pathways fit into the broader field, McSCs seem to fall within the ranks of other tissue SC types such as HF, mesenchymal, and intestinal SCs, which also use the BMP pathway to coordinate differentiation (Adam et al., 2018; Beumer and Clevers, 2021; Chen et al., 2012; Genander et al., 2014; He et al., 2004). For instance, to promote differentiation and restrain proliferation, intestinal SCs undergo a switch from WNT to BMP signaling (Beumer and Clevers, 2021; He et al., 2004), whereas in McSCs, BMP seems to both promote differentiation, suppress proliferation, but also enhance WNT signaling through LEF1. Interestingly, in hippocampal neural SCs, BMP and WNT signaling also seem to converge at the level of LEF1 (Armenteros et al., 2018). These similarities raise questions about the role of other more universal signaling pathways associated with SC differentiation, particularly SHH signaling.

4.2 Outstanding questions: unraveling the complex mechanisms up- and downstream of BMPR1a in the tissue microenvironment

My results suggest that signaling downstream of BMPR1a leads to SMAD-mediated upregulation of Lef1 mRNA and nuclear accumulation of LEF1 protein. While MITF is critical in promoting differentiation and appears to cooperate with LEF1 to do so, given that Mitf mRNA is not affected in Bmpr1a null cells, it is still not clear how BMP signaling induces accumulation of nuclear MITF post-transcriptionally. This is somewhat surprising, as LEF1 has been reported to activate transcription of Mitf-M in human cultured melanocytes (Takeda et al., 2002). In any case, in Lef1 null cells, nuclear MITF is diminished, supporting interplay between these two factors. Previous studies have provided some possible avenues for how this coregulation might occur. For instance, LEF1 (but not TCF1) can physically interact with MITF/TFE3 TFs (Yasumoto et al., 2002). Perhaps such interactions might induce stabilization and nuclear localization of MITF. Alternatively, BMP-induced LEF1 might medicate enhanced WNT signaling, which has been
shown to stabilize MITF, driving upregulation of genes involved in multivesicular body biogenesis. These bodies further enhance WNT by sequestering β-catenin destruction complex proteins (Ploper et al., 2015).

Furthermore, BMPR1a phosphorylation can trigger both canonical and noncanonical signaling cascades, with different BMP ligands and receptor combinations activating different downstream mediators. It seems likely that in vivo, both arms are this cascade are stimulated simultaneously. While *Lef1* upregulation appears to occur through canonical BMP-SMAD signaling, MITF levels might be controlled via SMAD-independent noncanonical pathways, such as p38-MAPK, which can phosphorylate MITF in osteoclasts (Mansky et al., 2002). In cultured human melanocytes, BMP6 signals through p38 to induce melanogenesis (Singh et al., 2012). At the same time, this paper also demonstrated that BMP4 reduces melanin production, making it difficult to predict what the role of this signaling pathway would be in the native HF niche and highlighting the importance of complementary in vivo studies.

Reflecting further on the report by Singh et al. (2012) demonstrating the anti-melanogenic effect of BMP4 in culture, it is intriguing that I find that BMP4 (and BMP2) both induce upregulation of *Lef1*. While WNT signaling is critical for melanocyte differentiation (Rabanni et al., 2011), it is not yet completely clear to what extent reduced nuclear LEF1 drives the BMPR1a loss-of-function phenotype. While LEF1/TCF family motifs are enriched in differentiation-enhanced ATAC peaks (Fig. 23A-B), it is possible that other TCF factors may be able to act in a functionally redundant manner. The *Lef1* KO mice do appear to have reduced hair pigmentation, but a conditional lineage-specific ablation approach would be ideal. To this end, I have been generating a *TyrCreER Lef1floxed* (Zhou and Xue, 2012) *R26YFPfloxed* strain to investigate whether the hair graying phenotype is recapitulated. Skin from these mice will similarly be investigated
with IMF, and YFP+ cells can be FACS purified to assess changes in transcription. To more conclusively define LEF1 targets, future ChIP-seq studies performed in vivo with techniques optimized for low cell input such as CUT&RUN (Skene and Henikoff, 2017) would be informative. One possibility to explore would be that LEF1 mediates target genes involved in proliferation. This fits with the finding that BMP4 (which in my hands upregulates Lef1) in downregulated during UV exposure (Singh et al., 2012). Perhaps this signal restrains proliferation in the HF bulb, but is downregulated upon UV in the epidermis to allow melanocytes to both proliferate and continue to produce melanin.

Finally, there is the question of which cells in the HF niche are providing the BMP ligands that McCP and melanocytes respond to during anagen. Autocrine signaling may be occurring, as McCP and melanocytes express Bmp2 and Bmp7 by scRNA-seq (Fig. 8C-D), preferential ligands of BMPR1a (Wang et al., 2014). However, the skin is rich in BMPs emanating from several compartments. One immediate possible source is the HF matrix TACs, which would make sense in terms of coordinating the differentiation of these two SCs compartments so that pigment could be transferred to hair cells at the appropriate time. BMP also directly regulates a cohort of hair-specific genes in the proliferative progenitors of the hair shaft (Adam et al., 2018; Genander et al., 2014). Another candidate would be the Krox20+ cells specifically, as these also provide SCF to promote melanocyte maturation and maintenance (Liao et al., 2017). BMPs also appear to be elevated at the apical region of the DP where proliferative precursors of both the hair shaft and the melanocytes reside (Yang et al., 2017). While it seems most likely that BMPs acting on the melanocyte lineage come from within the HF, other conceivable sources include the dermis, which exhibits cyclic expression of BMP2 and BMP4 or the adipose tissue that expresses BMP2 (Plikus et al., 2008), to which the anagen follicles gain proximity during downgrowth. In all probability,
several niche cell types producing different BMPs and extracellular regulators and the convergence of both canonical and noncanonical pathways with players like WNT converge to determine the outcome of BMPR1a signaling in the native microenvironment.

4.3 Future Directions

Studies investigating both specific pathways using genetic mouse models and global profiling methods in the melanocyte lineage in the HF have been critical for unraveling how McSCs transition through different cellular states in homeostasis. These in turn raise intriguing questions about how these genes and pathways act in phenotypic variation, aging, and diseases such as cancer. Pathways that regulate SC behavior often come into play in contexts outside of normal tissue regeneration. For example, the critical differentiation-promoting pathways MC1R and WNT/β-catenin are also necessary for McSC migration out of the HF niche to the epidermis during wounding (Chou et al., 2013; Sun et al., 2018). In melanoma, variants in MC1R, especially those resulting in fair skin and red hair, are associated with greater risk of developing cancer due not only to reduced pigmentation and UV-protection, but also because pheomelanin itself has carcinogenic properties (Mitra et al., 2012). Similarly, while its impacts on melanoma appear to be highly context-dependent, WNT/β-catenin is also a key player in melanoma (Gajos-Michniewicz and Czyz, 2020). Aging encompasses cumulative stress, injury, and inflammation over the course of an organism’s lifetime, on top of homeostatic tissue demands. Here, I discuss three exciting avenues of inquiry that stem from my thesis work, exploring the possible roles of BMP signaling in color variation, age-related McSC decline, and melanoma.
4.3.1 Potential mediators of phenotypic diversity in hair pigmentation and patterning

The gray hair phenotype elicited by loss of Bmpr1a (Fig. 3.3 A) raises the question of whether this pathway might be altered in nature to achieve different skin and coat colors or contribute to pattern formation. In HFs, this could be accomplished through downregulation of BMPR1a cell surface expression during differentiation and or by differential ligand or inhibitor (i.e. noggin) production in the microenvironment. Whether BMPR1a signaling might affect melanoblast migration during embryonic development in some species, thus affecting pigmentation and patterning (as in SCF/c-KIT and EDN3/EDNRB mutant mice), is another possibility. Indeed, several of the genes upregulated in Bmpr1a null cells were related to migration and cell adhesion (Fig. 3.6 B-C). At the same time, migration to the epidermis in adult mice in response to wounding or UV exposure is dependent on MC1R (Chou et al., 2013), which is downregulated in Bmpr1a cKO mice. Therefore, there are several potential mechanisms by which BMP could elicit phenotypic diversity in hair, fur, skin, and feathers.

While thoroughly investigating the status of epidermal melanocytes in the Bmpr1a cKO mice was beyond the scope of my study, I did not observe overt phenotypic differences in the ears, feet, or tail where these populations reside. There is reason to believe that such effects might only be seen upon UV exposure to enhance their basal activity. Singh et al. (2012) showed that upon UV exposure, human cultured melanocytes upregulate Bmpr1a, Bmpr1b, Bmpr-II, and Bmp6. Furthermore, their work suggests that different classes of BMPs can have opposing effects on melanogenesis, again highlighting the possibility of differential regulation of this pathway to achieve diverse colors and patterns.
4.3.2 Elevated BMP levels in aging skin and potential implications for hair graying

This deeper understanding of the role of BMP signaling in McSC differentiation into melanocytes in the HF raises an intriguing question: could perturbed BMP signaling occur throughout aging and contribute to hair graying? This is especially interesting in light of previous work in Fuchs Lab demonstrating that the production of BMPs in the skin increases with age in mice (Keyes et al., 2013). For aged HFSCs in the bulge, this results in increased pSMAD1/5/9 activity and longer periods of quiescence, leading to reduced hair growth (Keyes et al., 2013). Given this changing microenvironment and the importance of BMPR1a signaling in promoting melanocyte lineage differentiation under normal conditions, it is tempting to speculate that BMP might contribute to age-related hair graying. Indeed, while limited, there is some evidence linking increased WNT/β-catenin signaling in the skin to hair graying in mice (Zhang et al., 2017). These findings further suggest that pathways that normally regulate McSC lineage progression can be disrupted in age.

Hair graying can result from depletion or dysfunction of McSCs or McCP/melanocyte in the hair bulb. In actuality, a combination of issues might come into play as the long-lived McSCs and surrounding HF and skin microenvironment change during aging. In the case of BMP signaling, one might hypothesize that age-related hair graying might result from 1) increased BMP signaling causing ectopic activation of this pathway in bulge McSCs, driving their depletion and subsequent loss of their differentiating progeny, and or from 2) the impaired ability of McSC progeny to respond to BMP signaling, resulting in their failure to fully differentiate into pigment-producing cells, causing loss of color in the growing hair. These mechanisms could be investigated in mice, who like humans, exhibit age-associated hair graying; Zhang et al., (2017) observed graying at 34 months of age in C57/BL6 mice. Signs of ectopic activation of BMP signaling in
McSCs might result in pSMAD⁺ DCT⁺ McSCs and or differentiated (melanogenic) melanocytes in the bulge area. Impaired ability of differentiating progeny in aging follicles might elicit a phenotype reminiscent of Bmpr1a cKO mice, where anagen follicles with gray/white hair show DCT⁺ differentiating progeny in the hair bulb without nuclear pSMAD1/5/9, LEF1, or MITF. Cells from both conditions could be FACS purified to assess changes in receptor, BMP ligand, and downstream target gene expression in aged versus young adult mice.

Such studies in McSCs and their lineage and their response to BMPs throughout aging might shed light on disruptions that occur in other tissue SC types where BMP signaling is active. Furthermore, age-related deterioration of melanocyte function lessens their ability to produce melanin and provide UV protection, promoting epidermal aging and increasing the risk of skin cancer, rendering such investigations all the more important. If elevated BMP signaling in age does negatively impact McSCs (as has been shown for HFSCs), one could conceive of potentially using topical BMP inhibitors or intradermal injection of recombinant protein inhibitors as an anti-aging strategy to boost both hair growth and pigmentation.

**4.3.3 BMPR1a as a possible switchboard for melanoma cells**

Arguably one of the most exciting avenues opened up by this work are questions pertaining to how BMP might impact transformed cells of this lineage: melanoma. Not surprisingly, both pro- and anti-tumorigenic effects of this pathway have been described, but BMP has often been linked to melanoma invasiveness and migration (Braig and Bosserhoff, 2013; Gramann et al., 2019; Hsu et al., 2005; Rothhamer et al., 2005; Sinnberg et al., 2018; Venkatesan et al., 2018). For example, BMP2 is highly expressed in invasive melanoma cell lines, is thought to promote epithelial-mesenchymal transition, and is elevated in the blood of stage IV melanoma patients.
(Sinnberg et al., 2018). Intriguingly, recent work in zebrafish melanoma has indicated that ligand-activated BMP signaling actually inhibits differentiation and cancer cell death (Venkatesan et al., 2018). This group further demonstrated that GDF6 is expressed in melanoma (but not in normal melanocytes) and acts through BMP receptors to maintain a neural crest signature through MITF inhibition. Furthermore, GDF6 expression in melanoma is inversely correlated with patient survival (Gramann et al., 2019).

However, my work demonstrates that in the mammalian physiological context, loss of BMP signaling suspends the melanocyte lineage in a committed progenitor-like state, where cells are proliferative but unable to fully differentiate. Therefore, one might hypothesize that melanoma cells could exploit this pathway to progress in malignancy, and that in certain contexts, being refractory to BMPR1a signaling could promote activated McSC/McCP-like characteristics. Perhaps in premalignant cells or early in tumor initiation, enhanced BMPR1a signaling might help to push cells towards a more differentiated (MITF$^{\text{high}}$ LEF1$^{\text{high}}$) and less proliferative state, blunting progression. As the tumor advances, however, cells could downregulate BMPR1a or its activating ligands to achieve an MITF$^{\text{low-medium}}$ LEF1$^{\text{low-medium}}$ state with less melanin production, more proliferation, and enhanced expression of stem and migratory genes. Support for such a scenario comes from familial cancer syndromes caused by germline mutations in Bmpr1a, such as Juvenile Polyposis and Cowden disease (Hsu et al., 2005; Zhou et al., 2001). These syndromes are characterized by benign overgrowths and increased susceptibility to cancer. Zhou et al. (2001) found that many Bmpr1a mutations in these patients were predicted to result in truncated receptors and diminished BMP signaling, reminiscent of the over-proliferation of McCP in Bmpr1a cKO mice. Therefore, BMPR1a might have both oncogenic and tumor suppressive roles, similar to reports where both pro- and anti-melanogenic or differentiation effects have been observed,
depending on the context (Bilodeau et al., 2001; Han et al., 2012; Jin et al., 2001; Kawakami et al., 2008; Park et al., 2009; Sharov et al., 2005; Singh et al., 2012; Yaar et al., 2006; Yang et al., 2014).

Indeed, related superfamily member TGF-β can enhance the proliferation of tumor-initiating cells when signaling is silenced, while simultaneously playing a powerful counter role in promoting invasion and metastasis (David et al., 2016; David and Massague, 2018; Guasch et al., 2007; Oshimori et al., 2015; Su et al., 2020). It is tempting to speculate that in melanoma, the juncture between immature McCP and differentiated melanocytes might become plastic through cells flipping their reliance on BMP signaling, depending on the stage of disease progression and particular microenvironment.

Future studies in mouse models of melanoma (to complement those performed in zebrafish) may illuminate whether mechanisms involving BMPR1a might be at work. If so, melanoma might conceivably be treated with BMP receptor agonists or antagonists. At the same time, in addition to BMPR1a and its downstream signaling, my extensive scRNA-seq of the lineage, in particular that of quiescent and activated McSCs and McCP, provides a wide range of genes and pathways whose role in both normal lineage and melanoma biology is yet to be determined.
CHAPTER 5:
MATERIALS AND METHODS
Mice

All mice were maintained in The Rockefeller University’s Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility, the Comparative Bioscience Center. All mouse procedures were performed under Institutional Animal Care and Use Committee (IACUC)-approved laboratory protocols.

*Dct*-eGFP mice (Tg(Dct-EGFP)NY2Gsat/Mmucd, RRID:MMRRC_032849-UCD) were obtained from Mutant Mouse Resource and Research Center (MMRRC) at University of California at Davis and was donated to the MMRRC by Dr. Nathaniel Heintz of The Rockefeller University (GENSAT) (Gong et al., 2003). *Lef1*-RFP mice were generated previously in the Fuchs Lab by Rendl, et al. (2005). *Dct*-eGFP (mixed C57BL6/J, JAX#000664) males were crossed to CD1 females (CRL, Strain 022) to increase litter size and improve maternal care for mice used in qMcSC and aMcSC studies (*Dct*-eGFP*" only). Background strain was kept as consistent as possible in generating *Dct*-eGFP*+ Lef1*-RFP*+ mice, mating *Dct*-eGFP (mixed C57BL6/J) males with *Lef1*-RFP (CD1) females. *TyrCreER* (JAX#012328) (Bosenberg et al., 2006) mice were purchased from Jackson Laboratories and mated to in house colonies of *Bmpr1a floxed* (Mishina et al., 2002) *R26YFP floxed* (JAX#006148) (Srinivas et al., 2001) (mixed C57BL/6) mice. The *Lef1* KO strain (C57BL/6J) was obtained from Dr. Rudolf Grosschedl (Kratochwil et al., 1996). *Ctnnb1 floxed* (JAX#004152) (Brault et al., 2001) (C57BL/6J) were purchased from Jackson Laboratories.

For RNA and ATAC-seq experiments and IMF quantifications, male mice were used to eliminate sex-related differences, minimize variability in the hair cycle, and capture more cells per experiment. Male and female mice were used for IMF analysis (sex-matched for comparison), and female mice were used for *TyrCreER Bmpr1a floxed R26YFP floxed* 12 day post-waxing RT-qPCR.
Photographs of the gross phenotypes of mice were taken with iPhone 6, 7 Plus, and 11 Pro Max cameras and brightened in Fiji/ImageJ as necessary. Genotyping was performed using the primers listed in Table 5.1 below.

**Table 5.1 Genotyping Primer List**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’ &gt; 3’</th>
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<tr>
<td>β-catenin 1</td>
<td>AAGGTAGAGTGATGAAAGGTTGTT</td>
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<tr>
<td>β-catenin 2</td>
<td>CACCATGTCTCTGTCTATTCC</td>
</tr>
<tr>
<td>β-catenin 3</td>
<td>TACACTATTGAATCACAGGGACTT</td>
</tr>
<tr>
<td>Bmpr1a Fx2 Forward</td>
<td>GCAGCTGCTGCTGCAGCCTCC</td>
</tr>
<tr>
<td>Bmpr1a Fx4 Reverse</td>
<td>TGGCTACAATTTGCTCCTCATGC</td>
</tr>
<tr>
<td>Dct(32849) F Forward</td>
<td>GTGGGAATTTTGAGAGAGAGGAAAG</td>
</tr>
<tr>
<td>GFPR2 Reverse</td>
<td>TAGCGGCTGAAGCACTGCA</td>
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<tr>
<td>Lef1-RFP Forward</td>
<td>CACCTTGTGATACCCTTCTG</td>
</tr>
<tr>
<td>Lef1-RFP Reverse</td>
<td>CCCTGAAAACCTTTGCCCCCTC</td>
</tr>
<tr>
<td>Neo A</td>
<td>TGGAGAGGCTATTCGGCTATGC</td>
</tr>
<tr>
<td>Neo B</td>
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<tr>
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<tr>
<td>TyrCreER Reverse</td>
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<tr>
<td>Rosa26YFP common</td>
<td>AAAAGTCGCTCTGATTTGTATAT</td>
</tr>
<tr>
<td>Rosa26YFP mutant</td>
<td>AAGACCGCGAAGAGGTTTGTC</td>
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<tr>
<td>Rosa26YFP WT</td>
<td>GAGACCGGGAGAATGGATATG</td>
</tr>
</tbody>
</table>

Animal procedures

**EdU incorporation.** To assess McSC proliferation by IMF, two 100 uL doses of 5 mg/mL 5-Ethynyl-2’-deoxyuridine (EdU) were administered by intraperitoneal injection (IP). Mice were euthanized and skin was collected 24 hours after the first dose of EdU. To quantify proliferation of McSCs and their differentiating progeny in the hair bulb in **TyrCreER Bmpr1a^floxed R26YFP^floxed** mice one week after waxing, animals were given 100-150 uL EdU by IP 4 hours prior to sacrifice and skin collection.

**Tamoxifen treatment.** Starting at P21 (weaning age), **TyrCreER Bmpr1a^floxed R26YFP^floxed** and **TyrCreER Ctnnb1^floxed R26YFP^floxed** mice were given 50uL 2% tamoxifen dissolved in Sigma corn oil by IP for seven consecutive days. In second telogen (P60-75), back skin was waxed under
isoflurane anesthesia. Buprenorphine was given for pain management, and nails were clipped to minimize scratching. To prevent fighting and injury of waxed skin, male mice were singly housed after the procedure until the time of euthanasia.

**Fluorescence-activated Cell Sorting**

To obtain single cell suspensions for FACS purification, mouse back skin was dissected and subjected to chemical and mechanical digestion. Telogen (P58-80 for qMcSCs) and anagen I-II (P21 for aMcSCs) skin was scraped with a dull scalpel to remove excess fat prior to rotating incubation in trypsin-EDTA (Gibco) hair side up at 37°C for 45-60 minutes. Skin was then scraped with a dull scalpel against the direction of hair growth to release cells in the HFs. Anagen skin (P9-10 or 1 week or 12 days post waxing) was digested hair side up in 0.25% collagenase (Sigma) in HBSS (Gibco) at 37°C for 25-30 minutes. DNAse (Roche, 1:200) and 5mM MgCl₂ was sometimes added to anagen skin preparations to minimize cell clumping. The skin was then scraped from the dermal side to preferentially release cells in the hair bulb. The suspensions were quenched with cold FACS buffer (5% FBS in 1X PBS), filtered, spun down, and incubated for 25 minutes on ice with primary antibodies. All antibodies used are listed in Table 5.2. For biotin-conjugated antibodies, cells were washed and incubated with streptavidin secondary antibody for 5-10 minutes on ice.
### Table 5.2 FACS Antibody List

<table>
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<tr>
<th>Antibody</th>
<th>Company, Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly-6A/E (Sca-1) PerCP-Cy5.5</td>
<td>eBioscience, 45-5981-82</td>
<td>1:1000</td>
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<td>Ly-6A/E (Sca-1) APC-Cy7</td>
<td>BioLegend, 108126</td>
<td>1:1000</td>
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<tr>
<td>CD34 eFluor660</td>
<td>eBioscience, 50-0341</td>
<td>1:100</td>
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<tr>
<td>CD49f (α-6) BV421</td>
<td>BioLegend, 313624</td>
<td>1:100</td>
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<tr>
<td>CD49F (α-6) PE-Cy-7</td>
<td>BioLegend, 313621</td>
<td>1:100</td>
</tr>
<tr>
<td>CD117 (c-KIT) PE-Cy-7</td>
<td>eBioscience, 25-1171-82</td>
<td>1:100</td>
</tr>
<tr>
<td>Biotin-CD140a</td>
<td>BioLegend, 135910</td>
<td>1:150</td>
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<tr>
<td>Biotin-CD31</td>
<td>BioLegend, 102404</td>
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<td>Biotin-CD45</td>
<td>BD, 553077</td>
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<tr>
<td>Streptavidin PE</td>
<td>eBioscience, 12-4317</td>
<td>1:1500</td>
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<tr>
<td>Streptavidin APC-Cy7</td>
<td>BD, 554063</td>
<td>1:500</td>
</tr>
</tbody>
</table>

DAPI was used for live/dead cell exclusion. For bulk RNA-seq and RT-qPCR, cells were sorted with a 70 um nozzle directly into TRIzol (ThermoFisher). For scRNA-seq, cells were sorted with a 100 um nozzle into 96 well plates, gating more conservatively to minimize the capture of contaminating cell types. Cells were sorted by the Flow Cytometry Resource Center of The Rockefeller University. Flow cytometry plots were generated using FlowJo to illustrate the strategies used to isolate each cell population. Manual compensation was performed for presentation of flow plots.

**Immunofluorescence**

To prepare tissue for IMF analysis, mouse back skin was prefixed in 4% paraformaldehyde in PBS immediately after dissection for 30 minutes at room temperature or 1-2 hours at 4°C. Tissue was then washed with 1X PBS and incubated while rotating in 30% sucrose in PBS at 4°C overnight before embedding in OCT Compound. Frozen blocks were sectioned (10-18µm thickness) on a Leica cryostat and mounted on SuperFrost Plus slides (Thermo Fisher). Unstained slides were stored at -20°C until further processing.
Slides were dried at room temperature before blocking for at least an hour in IMF staining buffer consisting of 5% normal donkey serum, 1% bovine serum albumin, 2% fish gelatin and 0.3% Triton X-100 in PBS. For TYR and MITF IMF, slides were additionally fixed in cold methanol at 20°C for 20 minutes before blocking. Slides were incubated with primary antibodies diluted in IMF staining buffer (listed in Table 5.3) at 4°C overnight and washed three times with PBS prior to incubation with secondary antibodies (Alexa Fluor-RRX, 488, or 647-conjugated, Life Technologies at 1:500 dilution) for an hour at room temperature. For EdU, the Click-iT EdU Alexa Fluor 647 Imaging Kit (C10340) was used before proceeding to secondary antibody staining. Slides were washed another three times before mounting with ProLong Antifade Gold with DAPI Mountant (Thermo Fisher). Counterstaining with DAPI in PBS was sometimes performed to enhance nuclear signal.

Table 5.3 Primary Immunofluorescence Antibody List

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<th>Antibody</th>
<th>Company, Catalogue #</th>
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<td>DCT (TYRP2, D-18)</td>
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<td>TYRP1</td>
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<td>Tyrosinase (PEP7)</td>
<td>Made by V.J. Hearing</td>
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<td>CD104 (integrin β-4)</td>
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<td>PMEL (Anti-Melanoma gp100 [EP4863(2)])</td>
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<td>GFP</td>
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<td>RFP</td>
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<tr>
<td>(Ser463/465)/ Smad9 (Ser465/467)</td>
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<td>(D5B10)</td>
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<td>MITF</td>
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<tr>
<td>LEF1</td>
<td>Made by Fuchs Lab</td>
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**Immunofluorescence Microscopy**

IMF microscopy images were acquired with an Axio Observer.Z1 epifluorescence microscope with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics) and with an ApoTome.2 (Carl Zeiss) slider using 20, 40, or 63X objectives and Zen software (Carl Zeiss).
Differential interference contrast (DIC) brightfield images were taken on the Axio Observer.Z1 using the Axiocam 305 camera (Zeiss). Images were processed by channel using Fiji/ImageJ using Brightness/Contrast where appropriate. Images with Z-stacks were displayed as max intensity projections. Optical density (OD) was determined with MetaMorph software (Molecular Devices), where \( \text{OD} = \log_{10} \left( \frac{1}{\text{transmittance}} \right) \), and transmittance=transmitted light/incident light. Measurements were made of a single plane of DIC bright field image with Integrated Morphometry Analysis within a freehand drawn region outlining the DCT-stained region (based on maximum intensity projection).

**Electron microscopy**

Skin samples were fixed in 2% glutaraldehyde, 4% PFA, and 2 mM CaCl2 in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature for >1 hour, post-fixed in 1% osmium tetroxide, and processed for Epon embedding. Ultrathin sections (60–65 nm) were counterstained with uranyl acetate and lead citrate. Images were taken with a transmission electron microscope (Tecnai G2-12 FEI) equipped with a digital camera (AMT BioSprint29). For quantification, melanosomes were counted within hair bulb McSC progeny areas \( \geq 10 \mu m^2 \). These areas were outlined by hand by H. Amalia Pasolli, an expert in EM analysis of the skin. Melanosomes were scored as immature by the presence of striations/translucency and as mature (stage IV) if opaque. Measurements were expressed as melanosome number/cell area captured.

**Cell Culture**

The Melan-a2 mouse melanocyte cell line was generated by Sviderskaya, et al. (1995) and purchased from the Wellcome Trust Functional Genomics Cell Bank of St George's, University of London (http://anatomy.sgul.ac.uk/pages/). Cells were cultured in RPMI 1640 with L-glutamine supplemented with 10% FBS, 5% Penicillin-Streptomycin, 200 pM cholera toxin, and 200 nM
phorbol 12-myristate 13-acetate and grown at 7.5% CO₂. Cells were plated for experiments 24 hours before treatment in normal growth media, then serum starved at 0.5% FBS during the course of treatment (10 ng/mL recombinant human/murine/rat BMP2, murine BMP4, or human BMP6 (Peprotech) and or 250 nM ALK inhibitor K02288 (Tocris) in DMSO).

To generate a Bmpr1a KO Melan-a2 cell line with CRISPR, we first made a clonal parental cell line from a single Melan-a2 cell to minimize experimental variability. Predesigned gRNA was purchased from Integrated DNA Technologies (IDT) (control non-targeting and DesignID:Mm.Cas9.BMPR1A.1.AB Bmpr1a, Exon 9, 5’-CAUGACGCAUAACACCGUCGUUUUAGAGCUAUGCU-3’). Cells were transfected with Lipofectamine RNAiMAX (ThermoFisher) to deliver ribonucleoproteins (RNPs) consisting of duplexed gRNA and tracrRNA-Atto550 with Alt-R S.p. Cas9 Nuclease V3 (IDT). Culture media was changed 12-24 hours post transfection, and then genomic DNA was extracted with QuickExtract (Lucigen). The cut site region was PCR amplified, and cutting efficiency was tested by T7 endonuclease assay (New England Biolabs). To generate the Bmpr1a KO cell line, the parental clonal cell line was transfected and single cells were sorted into 96 well plates from which cell lines were grown. Gene KO was confirmed by MiSeq and the rgenome.net Cas-analyzer tool. The relevant primers are listed in Table 5.4 below.
Table 5.4 Primers used for Bmpr1a CRISPR KO in Vitro

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Purpose</th>
<th>Sequence 5’ &gt; 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sg_Ex9_CutSite_F1</td>
<td>Bmpr1a cut site PCR (T7 assay)</td>
<td>GGACTGTGTCGGATCAGCTAGG</td>
</tr>
<tr>
<td>Sg_Ex9_CutSite_R1</td>
<td>Bmpr1a cut site PCR (T7 assay)</td>
<td>TGTGTACCATCACACCCACTCA</td>
</tr>
<tr>
<td>BH_Seq_CutSite_F1</td>
<td>MiSeq validation of Bmpr1a KO (allows for barcoding, library prep)</td>
<td>TCGTCGGCAGCGTCAGATGTGTATAAAGAGACAGGGAGCTATTTGGTTATGCCAGGCTT</td>
</tr>
<tr>
<td>BH_Seq_CutSite_R1</td>
<td>MiSeq validation of Bmpr1a KO (allows for barcoding, library prep)</td>
<td>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAGCTACACAACCATTTGAATCCACA</td>
</tr>
</tbody>
</table>

RT-qPCR

Cultured cells were washed with PBS and harvested by resuspending in TRI Reagent LS (Sigma); FACS purified cells were sorted directly into TRI Reagent LS. RNA was purified using the Direct-zol RNA MicroPrep kit (Zymo Research) according to the kit protocol. cDNA was made with the SuperScript VILO cDNA Synthesis Kit (ThermoFisher) and diluted before RT-qPCR, which was performed using SYBR Green PCR master mix (ThermoFisher). Ppib2 was used as a housekeeping gene, and primer sequences are listed in Table 5.5.
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5' &gt; 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gapdh</em> Forward</td>
<td>GTCGTGGAGTCTACTGCTGGTCTTCAC</td>
</tr>
<tr>
<td><em>Gapdh</em> Reverse</td>
<td>GTTGTCATATTTTCTCGTGGTTCACACCC</td>
</tr>
<tr>
<td><em>Bmpr1a</em> Forward</td>
<td>GCTATTGCTCAGGACACTGC</td>
</tr>
<tr>
<td><em>Bmpr1a</em> Reverse</td>
<td>TGGTTTCTCCCTGATCATCCTT</td>
</tr>
<tr>
<td><em>Eps8</em> Forward</td>
<td>TCTTCACCACCCTATCCCCAG</td>
</tr>
<tr>
<td><em>Eps8</em> Reverse</td>
<td>CATCTTTCCGATCCAGCACGA</td>
</tr>
<tr>
<td><em>Id2</em> Forward</td>
<td>CTATCGTCAGCCTGCATCAC</td>
</tr>
<tr>
<td><em>Id2</em> Reverse</td>
<td>ATTCAGATGCTGCAAGGAC</td>
</tr>
<tr>
<td><em>Kif26b</em> Forward</td>
<td>TACACCATGATCGGAAGGGAC</td>
</tr>
<tr>
<td><em>Kif26b</em> Reverse</td>
<td>CTTGAACAGCCAAGAAATAGCAC</td>
</tr>
<tr>
<td><em>Lef1</em> Forward</td>
<td>CGCTAAAGGAGAGTGCAGCTA</td>
</tr>
<tr>
<td><em>Lef1</em> Reverse</td>
<td>GCTGTCTCTCTTCTGGTGCT</td>
</tr>
<tr>
<td><em>Mitf</em> Forward</td>
<td>ACTTTCCCTATCCCATCCAC</td>
</tr>
<tr>
<td><em>Mitf</em> Reverse</td>
<td>TGAGATCCAGAGTTCTGGTACA</td>
</tr>
<tr>
<td><em>Oca2</em> Forward</td>
<td>CCTGAACATACAGAAGTTTGCT</td>
</tr>
<tr>
<td><em>Oca2</em> Reverse</td>
<td>GAGCAGAGGGAGTGCTCTTCCTTA</td>
</tr>
<tr>
<td><em>Pax3</em> Forward</td>
<td>CCGGGGCAGAAATACCCAC</td>
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<tr>
<td><em>Pax3</em> Reverse</td>
<td>GCCGGTGATAAAATACCTCCCG</td>
</tr>
<tr>
<td><em>Ppib2</em> Forward</td>
<td>GTGAGCGCTTCCCAGATGAGA</td>
</tr>
<tr>
<td><em>Ppib2</em> Reverse</td>
<td>TGCCGGAGTCGACAATGATG</td>
</tr>
<tr>
<td><em>Rab38</em> Forward</td>
<td>GGGACATTGCTGGTCAGAAA</td>
</tr>
<tr>
<td><em>Rab38</em> Reverse</td>
<td>GGCTTACATTAGGAGCGGT</td>
</tr>
<tr>
<td><em>Slc26a2</em> Forward</td>
<td>AGAGACGGGGCTTTTGATTTT</td>
</tr>
<tr>
<td><em>Slc26a2</em> Reverse</td>
<td>GCCACCAACAGGATACCCAC</td>
</tr>
<tr>
<td><em>Vimentin</em> Forward</td>
<td>GAAATTGCAGGAGTGCTGGT</td>
</tr>
<tr>
<td><em>Vimentin</em> Reverse</td>
<td>TCCACCTTTCCGTTCAAGGTC</td>
</tr>
</tbody>
</table>
Western blotting

Cultured cells were washed in cold 1X PBS, lysed in cold RIPA Buffer (Millipore) supplemented with protease and phosphatase inhibitors (Roche), and harvested by scraping. Cells were lysed for 30 minutes on ice with vortexing, centrifuged at 4°C for 10 minutes, and supernatant lysate was collected. Protein concentration was determined by BCA Assay (Pierce) read on a BioTek plate reader using a bovine serum albumin standard curve. Up to 50µg protein was loaded on NuPAGE 4-12% Bis-Tris Gels (Invitrogen) and run for 1 hour at 200V in NuPAGE MOPS SDS Running Buffer (Invitrogen). Protein was transferred at 30V overnight at 4°C onto PVDF membranes in NuPage Transfer Buffer (Invitrogen) with 20% methanol. Membranes were blocked in PBS-based blocking buffer (Odyssey) for at least 1 hour at room temperature before incubating with primary antibodies overnight at 4°C in blocking buffer supplemented with Tween-20. Membranes were washed several times in PBS with 0.1% Tween-20 before incubating with fluorescent secondary antibodies in blocking buffer supplemented with Tween-20 and SDS. Membranes were washed again before imaging on an Odyssey CLx machine (LI-COR). The primary and secondary antibodies and the dilutions used are listed in Table 5.6.

**TABLE 5.6 Immunoblotting Antibody List**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company, Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad9 (Ser465/467) (D5B10)</td>
<td>Cell Signaling, #13820</td>
<td>1:1000</td>
</tr>
<tr>
<td>SMAD1/5/9</td>
<td>Invitrogen, #PA1-41079</td>
<td>1:1000</td>
</tr>
<tr>
<td>LEF1 (C12A5)</td>
<td>Cell Signaling, #2230</td>
<td>1:1000</td>
</tr>
<tr>
<td>MITF (D5G7V)</td>
<td>Cell Signaling, #12590</td>
<td>1:1000</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Invitrogen, #T5168</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Donkey anti-rabbit 800 (secondary)</td>
<td>LI-COR, #926-32213</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Donkey anti-mouse 680 (secondary)</td>
<td>LI-COR, # 926-68072</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>
**Bulk RNA-seq**

Cells were FACS purified into TRI Reagent LS, and total RNA was purified using the Direct-zol RNA MicroPrep kit (Zymo Research). For McSCs, cells from 4 male mice at P60 were pooled for Replicate 1, and cells from 4 male mice at P62 were pooled for Replicate 2. For differentiating McSC progeny in the hair bulb, cells from male P10 mice were also pooled: 3 mice for Replicate 1, 2 mice for Replicate 2, and 4 mice for Replicate 3. The Weill Cornell Medical College Genomic Core facility (New York, NY) performed quality control analysis and sequencing. Briefly, RNA quality was determined by Agilent 2100 Bioanalyzer, and cDNA libraries were prepared using the Illumina TrueSeq mRNA sample preparation kit (non-stranded, poly-A selection) and sequenced on an Illumina HiSeq 4000 instrument.

**Single cell cDNA synthesis and library preparation**

ScRNA-seq libraries were prepared using a slightly modified version of the Smart-seq2 protocol described by Picelli et al. (2014). Cells were FACS sorted into 96 well plates with 2µL lysis buffer containing Triton X-100, RNaseOUT (Invitrogen), Oligo dT30VN, dNTPs, and ERCC spike-ins. Plates were flash frozen in liquid nitrogen and stored at -80°C until further processing. Plates were then thawed on ice before lysing at 72°C for 3 minutes. The RNA was subject to reverse transcription (4U/µL Maxima H- transcriptase), template switching reaction, and whole transcriptome amplification. cDNA was purified with 7µL AMPure XP beads per well. To test for low quality libraries or empty wells, RT-qPCR for Gapdh was performed before proceeding. Illumina sequencing libraries were then prepared using the Nextera XT DNA library preparation kit (Illumina). After barcoding, samples were pooled and purified with MinElute PCR Purification Kit (Qiagen), then cleaned with 0.9X by volume AMPure XP beads (Beckman Coulter). Libraries were sequenced on an Illumina NextSeq 500 (75 bp paired-end read, mid-output).
ATAC library preparation

Cells were FACS purified using an 85µm nozzle for gentler sorting to improve viability. qMcSCs were isolated as described in Fig. 2.2 B from $Dct$-eGFP+ x CD1 2nd telogen skin at P60 (Replicate 1, cells from 3 male mice pooled) and P80 (Replicates 2 and 3, cells from 4 male mice pooled for each); differentiating progeny were isolated from $Dct$-eGFP+ (C57BL6/J background with some CD1) anagen skin at P9 (Replicate 1, cells from 1 male mouse, Replicates 2 and 3, cells from 2 male mice pooled for each) using the following FACS strategy: dump (CD31, CD45, CD140a), Sca-1−, CD34+, eGFP$^{\text{high}}$, CD117+ cells). Library preparation was performed as described (Buenrostro et al., 2013) with some modifications. Briefly, sorted cells were washed with cold PBS, pelleted, and resuspended in cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl$_2$, 0.1% IGEPAL CA-630). Buffer was removed after centrifugation, then samples were incubated in the transposition reaction at 37°C for 30 minutes (Illumina Nextera DNA Preparation Kit, using 10uL TDE1 enzyme). The reaction was terminated by adding Tagmentation Clean Up buffer consisting of 300 mM EDTA and 900 mM NaCl. DNA was purified with the Qiagen MiniElute PCR purification kit, then PCR amplified with barcode identifiers for 12-18 cycles, and the products taken at three cycling times were tested by D1000 Tape Station (Agilent). Optimal samples were then pooled and bead purified for sequencing on NextSeq High Output 75 single read (40 x 40 bp paired end).

Schematics

Some graphics from SMART Servier Medical Art (https://smart.servier.com) were used to make to make schematics. Other illustrations were created with Adobe Illustrator and Microsoft PowerPoint.
Quantification and Statistical Analyses

Statistical tests for microscopy quantifications and RT-qPCR were performed with GraphPad Prism 7. Column data was first subject to D’Agostino and Pearson normality testing. For data without normal distribution, two-tailed Mann-Whitney test was used to compare two groups, and Kruskal-Wallis test with Dunn’s with multiple comparisons was used to compare between more than two groups. For RT-qPCR, where n number was too low for normality testing, unpaired t test or ordinary one-way ANOVA with Dunnett’s multiple comparison tests were performed.

Bulk RNA-seq analysis

Sequence and transcript coordinates for mouse mm10 UCSC genome and gene models were retrieved from the Bioconductor Bsgenome.Mmusculus.UCSC.mm10 (version 1.4.0) and TxDb.Mmusculus.UCSC.mm10.knownGene (version 3.4.0) Bioconductor libraries, respectively. Read length was 51bp. Transcript expressions were calculated using the Salmon quantification software (version 0.8.2) (Patro et al., 2017) and gene expression levels as TPMs and counts were obtained using Tximport (version 1.8.0) (Soneson et al., 2015). Normalization and rlog transformation of raw read counts in genes were performed using DESeq2 (version 1.20.0) (Love et al., 2014). Variability between samples was assessed with hierarchical clustering and heat maps of between sample distances implemented in the Pheatmap R package (1.0.10) as described by Love et al. (2014), and sample similarity is expressed as Euclidean distance. Published HFSC RNA-seq data from Ge et al. (2017) was obtained from Gene Expression Omnibus (GEO) accession #GSE89928 (GSM2656733 CL_BuA and GSM2656734 CL_BuB).
Single cell RNA-seq analysis

Sequence and transcript coordinates for mouse release M23 (GRCm38.p6) genome and gene models were downloaded from GENCODE (https://www.gencodegenes.org/mouse/release_M23.html). Adaptors were trimmed from reads using Skewer (version 0.2.2). Sequencing reads were aligned to the mouse reference genome combined with sequences for ERCC spike-ins as artificial chromosomes using STAR (version 2.5.2a) (Dobin et al., 2013) with default parameters for paired-end reads. Transcript expressions were calculated with Salmon (version 0.14.1) (Patro et al., 2017), and gene expression levels as TPMs and counts were obtained with Tximport (version 1.12.3) (Soneson et al., 2015). TPMs were transformed to log2(TPM+1). For downstream analyses, cells with <1,250 genes detected per cell and genes expressed in <5% of the cell population were removed. Cells expressing lower levels of Dct (log2(TPM+1) <6) and or those expressing the HF lineage gene Krt15 (log2(TPM+1) >6) were excluded. After filtering, the number of cells in the dataset were as follows: qMcSCs n=104 cells (n=3 mice), aMcSCs n=193 cells (n=3 mice), McSC differentiating progeny (McCP/melanocytes) n=308 cells (n=2 mice), Bmpr1a WT control n=205 cells (n=1 mouse), Bmpr1a heterozygous n=304 cells (n=2 mice), and Bmpr1a null n=421 cells (n=2 mice).

Analyses and visualization of data were conducted in a Python environment built on the Numpy, SciPy, matplotlib, scikit-learn package and pandas libraries (Pedregosa et al., 2011). Batch effect variation between sequencing runs and biological replicates was assessed by examining the Euclidean distances between replicates within each hair cycle timepoint dataset versus across hair cycle timepoints, which showed closer relationships between replicates compared with variation between biologically distinct collection timepoints. PCA analysis of the only dataset with some evidence of replicate-specific clustering patterns (P10 anagen VI) captured
biologically relevant differences in gene expression separating these replicates, suggesting that these differences likely represent true biological variation between animals in this highly dynamic differentiating population, rather than technical batch effects.

To distinguish biological variability in gene expression from technical noise, a statistical model for identifying highly variable genes compared to ERCC spike-ins as described by Brennecke et al. (2013) was implemented. A custom script based on the methodology described by Brennecke et al., 2013 (in R version 3.6.1) was used to identify those genes with variation at least 10% above the technical variation with FDR less than 0.1.

To visualize the data and identify clusters, the highly variable gene dataset was centered and scaled, and PCA was performed. A Jack-straw approach for the first 100 components (based on Seurat v3.1.1) (Stuart et al., 2019) was implemented in R to calculate a statistical significance for each PC, and components with p<0.05 were retained for downstream analysis. Significant PCs were used as input for non-linear dimensionality reduction, performed using UMAP implemented in scikit-learn. A graph-based clustering approach based on building a kNN graph and clustering with the Louvain algorithm (with k set to 1/5th of the dataset size, and resolution parameter of 1 x10^-4) was implemented. Euclidean distance in PCA space served as input for both UMAP generation and Louvain clustering. In second level analysis, the same strategy was used for dimensionality reduction, clustering, and visualization, with variable genes and PCs determined separately for each of the main cell clusters (C1, C2, C3).

For pseudotime analysis, the same strategy for dimensionality reduction and clustering of each dataset was used, principal graph based on the community analysis was constructed, and a pseudotime values for each cell was computed using Monocle 3 (version 0.2.0) (Cao et al., 2019; Trapnell et al., 2014). To identify genes that change as a function of pseudotime, the “graph_test()”
function of Monocle 3 was used to implement Moran’s I test. Genes were considered significantly changing here if they had a q-value<1.0x10^{-4}.

Differential gene expression was used to identify genes up- and downregulated in each cluster. Briefly, raw count matrices for the biologically variable genes within each dataset were used and applied to the DESeq2 package (version 1.24.0) (Love et al., 2014) using R. A negative binomial fit was used to model differential gene expression, the dataset was factored based on Louvain cluster assignments, and a threshold of 0.75 was used to construct Wald tests of significance. In general, genes were considered to be significantly differentially expressed if their log2(fold change) ≥|1| and p-adjusted value (q) ≤0.05. Lowly expressed differential genes (baseMean expression ≤5) were discarded from visualization and further analysis. Expression levels of specific genes of interest were visualized as log2(TPM+1) values on UMAP plots. The “stemness” and “differentiation” signature gene sets used to generate global scores were the top 100 up- or down-regulated differentially expressed genes between McSCs (C1 and C2) and McSC differentiating progeny (C3). The “AddModuleScore” function from Seurat v3.1.1 was used to calculate the average expression levels of each gene set on a single cell level, subtracted by the aggregated expression of control feature sets, as originally described in Tirosh et al. (2016).

GO analyses of differentially expressed genes were performed using PANTHER Tools Overrepresentation Test (version 14.0), and annotated using the GO Biological Process Complete list (Mi et al., 2019; Thomas et al., 2006), with significant associations calculated by the Fisher’s Exact test and multiple comparisons corrected for using FDR. Where indicated, GO analyses were also annotated using DAVID Bioinformatics Resources 6.8 “Functional Annotation,” “BP Direct” lists (Huang et al., 2009a; Huang et al., 2009b).
Heatmaps were constructed in a Python environment built on the Numpy, SciPy, matplotlib, scikit-learn package and pandas libraries (Pedregosa et al., 2011). For specific GO annotations, the corresponding *Mus musculus* gene lists were obtained from AmiGO 2 through the Gene Ontology Consortium. Each GO gene list was used to filter the biologically variable genes associated with the dataset to generate normalized expression matrices, and genes characteristic of cell clusters (expressed in >50% of the cluster cells) were used for visualization. Each expression matrix was scaled and centered, and hierarchical linkages between genes was calculated based on pairwise correlation distances using the Farthest Point Algorithm. The matplotlib extension Seaborn visualization library (version 0.9.1) was used to create heatmaps using the clustermap() function with the previously generated gene-based hierarchical linkages fed in.

BMP or canonical WNT target gene lists were culled from mouse literature (Table S8 of Infarinato et al., 2020); to be included, target genes had to be validated as direct targets of the signaling pathway in at least two mesenchymal or epithelial tissues, and be expressed in our datasets. Cell cycle stage specific gene lists were obtained from Macosko et al. (2015). To generate a global proliferation gene set score, an amalgamated list of cell cycle stage-specific genes was used. The “AddModuleScore” function from Seurat v3.1.1 was used to calculate the average expression levels of each gene set on a single cell level, subtracted by the aggregated expression of control feature sets, as originally described in Tirosh et al. (2016). Gene set scores for each cell are color-coded on UMAP visualizations of the data or on violin plots binned by sub-cluster cell identity.

Promoter analysis of differentially expressed genes

HOMER genome-wide motif predictions were used to scan for LEF1, MITF, and SMAD motifs within 2.5 kb upstream of the TSS of genes that were significantly changed with padj≤0.05,
baseMean≥5 in the Bmpr1a null versus control cluster. Motifs had to be found in at least one TSS to be considered positive, then genes were then grouped based on the presence or absence of motifs. The difference in fold change between two groups was tested by Wilcoxon test with the null hypothesis that there is no difference in the distribution between the test groups. The results were plotted with R (3.6.1) and ggplot2.

**ATAC-seq analysis**

ATAC-seq reads are aligned to the mm10 genome from the Bsgenome.Mmusculus.UCSC.mm10 Bioconductor package (version 1.4.0) using Rsubread's align method in paired-end mode with fragments between 1 to 5000 base-pairs considered properly paired (Liao et al., 2013). Normalized, fragment signal bigWigs were created using the rtracklayer package. Peak calls were made with MACS2 software in BAMPE mode (Feng J et al., 2012; Zhang et al., 2008) and sequences under these peaks used with the motifMatcher Bioconductor package (Schep et al., 2014) and JASPAR motif database (Fornes et al., 2020) to identify known motifs within ATAC-seq peaks. Differential ATAC-seq signal was identified using the DESeq2 package (Ross-Innes CS et al., 2012; Love et al., 2014) and enrichment for motifs identified using the Limma packages geneSetTest function (Ritchie et al., 2015). Integrative Genomics Viewer (IGV) was used for gene track and peak visualization.

**Data Availability**

The bulk and scRNA-seq and ATAC-seq and code generated during this study are available at GEO [GSE147299/ https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147299].
### APPENDIX

**Table 2.1** Select List of Differentially Expressed “Stemness” and “Differentiation” Genes from DESeq2 Comparison of C1 and C2 vs. C3 cells (see also Table S2 from Infarinato et al., 2020)

*All values shown to three decimal points, ranked by padj value

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>baseMean</th>
<th>log2FoldChange</th>
<th>stat</th>
<th>pvalue</th>
<th>padj</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arid5a</td>
<td>55.144</td>
<td>4.415</td>
<td>22.643</td>
<td>1.626E-113</td>
<td>9.691E-111</td>
</tr>
<tr>
<td>Lmna</td>
<td>146.456</td>
<td>3.407</td>
<td>22.635</td>
<td>1.948E-113</td>
<td>1.045E-110</td>
</tr>
<tr>
<td>Socs3</td>
<td>13.928</td>
<td>6.498</td>
<td>22.422</td>
<td>2.401E-111</td>
<td>1.171E-108</td>
</tr>
<tr>
<td>Col12a1</td>
<td>118.818</td>
<td>4.992</td>
<td>22.246</td>
<td>1.240E-109</td>
<td>5.544E-107</td>
</tr>
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<td>Gab1</td>
<td>25.657</td>
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<td>20.358</td>
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<td>Vim</td>
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<td>19.317</td>
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**Table 3.1:** Select List of Differentially Expressed Genes for *Bmpr1a* Null vs. Control Differentiating Progeny Cells from DESeq2 (see also Table S9 from Infarinato et al., 2020)

*All values shown to three decimal points, ranked by padj value*

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Table 3.2: DAVID GO terms from Gene Lists in Table 3.1 (see also Table S9 from Infarinato et al., 2020)

**DAVID GO Term Analysis Output: Statistical enrichment, GO Biological Process Complete**

**Control List**

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Table 3.3: List of Genes with Reduced Expression in *Bmpr1a* Null Differentiating Progeny Cells (padj $\leq 0.05$, baseMean $\geq 5$) with MITF and LEF1 Motifs Within Their Putative Promoter Region (see also Table S11 from Infarinato et al., 2020)

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Table 3.4: Motif Analysis of ATAC Peaks Increased in Differentiating Progeny Proximal to Genes Whose Expression is Diminished in *Bmpr1a* Null Cells (see also Table S14 from Infarinato et al., 2020)

*Values shown to three decimal points, where applicable

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REFERENCES


Nusse, R., Clevers, H. Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell* 169(6):985-999.


