ABSTRACT

Hair greying (canities) is one of the earliest, most visible ageing-associated phenomena, whose modulation by genetic, psychoemotional, oxidative, senescence-associated, metabolic and nutritional factors has long attracted skin biologists, dermatologists, and industry. Greying is of profound psychological and commercial relevance in increasingly ageing populations. In addition, the onset and perpetuation of defective melanin production in the human anagen hair follicle pigmentary unit (HFPU) provides a superb model for interrogating the molecular mechanisms of ageing in a complex human mini-organ, and greying-associated defects in bulge melanocyte stem cells (MSCs) represent an intriguing system of neural crest-derived stem cell senescence. Here, we emphasize that human greying invariably begins with the gradual decline in melanogenesis, including reduced tyrosinase activity, defective melanosome transfer and apoptosis of HFPU melanocytes, and is thus a primary event of the anagen hair bulb, not the bulge. Eventually, the bulge MSC pool becomes depleted as well, at which stage greying becomes largely irreversible. There is still no universally accepted model of human hair greying, and the extent of genetic contributions to greying remains unclear. However, oxidative damage likely is a crucial driver of greying via its disruption of HFPU melanocyte survival, MSC maintenance, and of the enzymatic apparatus of melanogenesis itself. While neuroendocrine factors [e.g. alpha melanocyte-stimulating hormone (α-MSH), adrenocorticotropic hormone (ACTH), β-endorphin, corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH)], and microphthalmia-associated transcription factor (MITF) are well-known regulators of human hair follicle melanocytes and melanogenesis, how exactly these and other factors [e.g. thyroid hormones, hepatocyte growth factor (HGF), P-cadherin, peripheral clock activity] modulate greying requires more detailed study. Other important open questions include how HFPU melanocytes age intrinsically, how psychoemotional stress impacts this process, and how current insights into the gerontobiology of the human HFPU can best be translated into retardation or reversal of greying.

Key words: ageing, graying, melanin, endocrine, senescence

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I. INTRODUCTION

Hair greying (referred to during ageing as ‘canities’) is one of the earliest and most visible indicators of ageing in humans. The social significance of greying persists across cultures, geographical locations, and ethnicities, alongside a now-widespread interest in its reversal (Trüeb & Tobin, 2010; Stenn, 2016). The modulation of greying by genetic, psychoemotional, oxidative, senescence-associated, (neuro-)endocrine, metabolic and nutritional factors has increasingly fascinated pigment and skin biologists, clinicians and industry. This is not least because the study of greying permits one to observe and interrogate gerontobiology of a uniquely accessible and experimentally tractable human mini-organ found throughout the human integument. Moreover, an increasing number of animal and human studies have identified grey hair as an age-independent serious extracutaneous pathology, including Alzheimer’s disease (Mendelsohn & Larrick, 2020), Parkinson’s disease (Jucevičiūtė et al., 2019) and cardiovascular disease (Elfaramawy et al., 2018), raising suspicion that hair greying may indeed act as an important indicator of systemic ageing-associated pathology. As we grow closer to explaining this poorly understood phenomenon, it is timely to revisit critically what is known about the biology of hair greying today.

This review focuses on the mosaically cycling human scalp hair follicles (HFs), whilst cautiously drawing from greying-related mechanistic concepts generated in studies of rapidly cycling mouse HFs (Mus musculus, L.) (Paus & Foitikz, 2004; Bernard, 2012, 2017; Oh et al., 2016; Zhang et al., 2020). Hair cycling pattern and rate, as well as other fundamental aspects of pigmentation and hair biology, differ between humans and other mammals, including mice (Stenn & Paus, 2001; Bernard, 2012). Therefore, caution is advised when extrapolating concepts on the biology of greying generated in mouse pelage HFs to human scalp HFs. Here, we purposely focus on the latter to highlight why human hair greying, besides its obvious relevance for human melanocyte biology, also represents an under-appreciated interdisciplinary model for human gerontology, immunology, perceived stress, stem cell, and neuroendocrine research.

Greying provides an instructive model for mouse and human tissue ageing, because the HF is highly susceptible to oxidative, inflammatory, nutritional and psychoemotional stressors (Peters et al., 2006; Peters, Arck & Paus, 2006a; Paus et al., 2014). Human scalp HFs are not only the largest HFs but also have a long growth phase (anagen) of the hair cycle, thus facilitating the interrogation of greying processes over several years. The study of long-term pigmentation loss is likely to promote our understanding of other systemic ageing controls (Peters et al., 2013). In addition, human scalp HFs can easily be obtained, dissected and organ-cultured under serum-free conditions following facelift or hair transplantation surgery. During ex vivo culture, pigment or white hair shaft production continues at almost the normal speed and anagen HF’s continue spontaneously to undergo their cyclic organ involution process called catagen (Philpott, Green & Kealey, 1990; Langen et al., 2015).

In this review, we first summarise the functional anatomy of human HF pigmentation and the strict coupling of hair shaft pigmentation by highly specialized melanocytes in the HF pigmentary unit (HFPU) to the active growth stage of HF cycling (anagen) (Fig. 1). This is followed by synthesizing key characteristics of human hair greying before focusing on documented and proposed mechanisms that underlie it. We expand this to discuss how functionally important controls of the human HFPU, such as neurohormones, thyroid hormones, adhesion molecules, growth factors and peripheral clock activity might impact upon the initiation of greying. Thereafter, we critically discuss the contribution of melanocyte stem cells (MSCs) to human hair greying, arguing that MSCs are late-comers to this event, which do not initiate but ultimately determine the potential for reversibility of greying. Further, we discuss the key role of oxidative stress in the greying process, in the context of dysregulated intracellular increases in free radicals, from both melanocyte-
intrinsic and extrinsic sources. Finally, we define key remaining questions in greying research.

II. HAIR CYCLE-COUPLED REGULATION OF PIGMENTATION AND GREYING

The human HF contains several sub-populations of melanocytes (Tobin, 2008a) that are distinct from those in the epidermis (Tobin & Bystryn, 1996). These are divided across different histologic compartments based on differentiation status, pigment content and function (Fig. 1). In pigmented Anagen VI HF's, melanocytes expressing active tyrosinase and its major product, the melanogenic intermediate dihydroxyphenylalanine (DOPA), are found in the basal infundibulum as well as in the hair matrix surrounding the mid to upper dermal papilla (those in the latter region constitute the HFPU) (Kukita & Fitzpatrick, 1955). Melanogenesis occurs within unique lysosome-derived, membrane-bound organelles termed 'melanosomes' that are transferred to the surrounding keratinocytes of the hair shaft via dendritic and
filopodial processes. The mechanism of transfer is not yet entirely clear, and is suspected to vary by tissue and species (Wu & Hammer, 2014). Human melanoblasts, or MSCs, are located in the bulge region of the human HF and are poorly or un-pigmented, containing few dendrites and low/no expression of glycoprotein 100 (gp100, also known as Pmel17/Silv) (Tobin & Bystryn, 1996). The hair bulb also contains an immature subpopulation of amelanotic (unpigmented) melanocytes in the proximal and peripheral regions (Tobin & Paus, 2001; Tobin & Kauser, 2005). Amelanotic melanocytes that are negative for DOPA oxidase activity of tyrosinase (but which may express inactive tyrosinase at the protein level) are also found in the mid to lower outer root sheath (Horikawa et al., 1996). It has been suggested that such amelanotic melanocytes are progenitors differentiated from MSCs (Tobin, 2008b). However, this role has yet to be confirmed.

HF pigmentation is strictly coupled to the hair cycle. In early anagen (I–II) no pigment is produced as the HF grows and differentiates following telogen (Oh et al., 2016). Subsequently, pigmentation is initiated during anagen III, and reaches its maximum in anagen VI HFs, both in mice and humans (Slominski, Paus & Costantino, 1991; Slominski &

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Fig 2. The cellular composition of the human hair follicle (HF) changes during greying. (A) Illustration summarizing the compositional differences between the melanocyte compartment of white, grey and pigmented human scalp HFs, based on Horikawa et al. (1996), Commo, Gaillard & Bernard (2004), Nishimura, Granter & Fisher (2005) and Tobin (2009). In fully pigmented follicles, the anagen hair bulb is rich in pigment-producing melanocytes, but also contains amelanotic melanocytes in its peripheral epithelium. During greying, pigmented melanocytes are lost from the bulb, although some ectopic pigmentation may be observed near the bulge (Nishimura, Granter & Fisher, 2005). Ultimately, glycoprotein 100 (gp100)-positive amelanotic melanocytes and presumptive melanocyte stem cells (MSCs) in the bulge are lost as well. Histochemically grey/white HFs demonstrate a lack, or very substantial reduction, of melanin (B, F), while immunofluorescence microscopy shows reduced expression of the pre-melanosome marker gp100 (C, G) and histochemically detectable tyrosinase activity (D, H). In the bulge, the number of gp100+ amelanotic melanocytes (see arrowheads) is reduced in grey hairs (E, I). CTS, connective tissue sheath; DAPI, 4',6-diamidino-2-phenylindole; DP, dermal papilla; HFPU, hair follicle pigmentary unit; HM, hair matrix; MITF low, microphthalmia-associated transcription factor (low = low expression); ORS, outer root sheath.
Paus, 1993; Slominski et al., 1994, 2005b; Müller-Röver et al., 2001; Oh et al., 2016) (Fig. 1). Current evidence (mostly from the mouse) suggests that in catagen, most differentiated HFPU melanocytes undergo apoptosis, while bulge MSCs survive, possibly along with some amelanotic melanoblasts and other melanocyte progenitors in the secondary hair germ (Tobin, 1998; Tobin et al., 1999; Commo & Bernard, 2000). This is equally evident in experimental induction of catagen using toxic agents such as cyclophosphamide and dexamethasone, as pioneered in mouse hair research (Slominski et al., 1996; Ermak & Slominski, 1997). Therefore, the prevailing view is that follicular MSCs that survive catagen contribute immature progenitors to replenish bulbar melanogenic melanocytes at the start of each new anagen phase (Tobin et al., 1999; Commo & Bernard, 2000; Nishimura et al., 2002). In humans, it is not yet clear which MSC sub-populations repopulate the HFPU during early anagen. However, corticotropin-releasing hormone (CRH) receptor+ melanocyte progenitor cells located in the most proximal hair matrix of human anagen HF (Ito et al., 2004b) could represent an alternative, bulge MSC-independent melanoblast pool. It is likely that these cells retreat into the secondary hair germ during catagen development, similar to HF progenitor cells (Geyfman et al., 2015; Oh et al., 2016; Pantelyev, 2018), and may have a key role in re-establishing the HFPU during the subsequent anagen. Probing this hypothesis is important for understanding both hair greying and the hair cycle.

The gradual nature of greying is evident upon histological investigation. Grey HF contain a reduced number of differentiated, i.e. melanogenically active, bulbar melanocytes (Commo, Gaillard & Bernard, 2004; Nishimura, Granter & Fisher, 2005; Tobin, 2009) (Fig. 2). Grey HF-resident melanocytes show vacuolization and incomplete melanization of melanosomes at the ultrastructural level (Orfano, Ruska & Mahrle, 1970), and contain aberrantly distributed melanosomes (Nordlund et al., 2008). Mature melanocytes may exhibit a dystrophic hypertrophic state during greying, accompanied by interrupted melanin transfer, and ectopic distribution of melanin in the dermal papilla and connective tissue sheath (Tobin, 2009), possibly due to phagocytosis of melanocytes or melanocyte fragments (Tobin, 1998). Some melanocytes in the HFPU become apoptotic [as observed via Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining], while those that remain exhibit reduced or abolished dendricity, and occasionally ectopic positioning in the proximal matrix (Arck et al., 2006).

Eventually, ‘white’ HF exhibit a complete loss of melanin transfer to the hair shaft, usually accompanied by the corresponding loss of all bulbar melanocytes. However, it is important to emphasize that even optically white scalp HFs may still contain a few isolated hair bulb melanocytes, some of which may engage in residual melanogenesis despite a lack of both dendritic morphology and transfer of melanin to the hair shaft (Arck et al., 2006) (Fig. 2). Amelanotic melanocytes present in the bulge, outer root sheath (ORS) and proximal matrix (Horikawa et al., 1996; Ito et al., 2004b; Commo, Gaillard & Bernard, 2004; Slominski et al., 2005b; Nishimura, Granter & Fisher, 2005) persist beyond the loss of the bulbar melanocytes, but are gradually lost over time. For instance, the number of gp100+ cells drops progressively in the ORS from grey and white HF, and in some cases gp100+ cells of white HF are completely absent (Horikawa et al., 1996; Commo, Gaillard & Bernard, 2004). Likewise, Nishimura, Granter & Fisher (2005) noted a progressive reduction and eventual loss and differentiation of speculative MSCs expressing low levels of microphthalmia-associated transcription factor (MITF; MITF<sup>low</sup>) in the bulge of human scalp HFs over the course of ageing.

Melanocyte sub-populations across the HF therefore appear to be differentially lost during ageing, with tyrosinase (DOPA) + melanocytes in the bulb disappearing first, followed by amelanotic melanocytes and presumptive MSCs in the bulge region, which remain unaffected for a long time after hair greying has already affected the HFPU. Currently, the stimuli that initiate melanocyte loss in the HFPU remain unclear. Certainly, the concept that failure of bulge MSCs to replenish the HFPU between each hair cycle is responsible for the onset of greying in human HF is frequently asserted (Jo et al., 2018; Clark & Deppmann, 2020), but still lacks supporting evidence. There is also no evidence that bulge MSCs replenish the human HFPU during the multi-year lifespan of anagen VI HFs (Tobin, 2011; Oh et al., 2016) (Fig. 1), despite the otherwise well-documented migration of these cells to the epidermis (Chou et al., 2013; Paus, 2013).

Thus, in light of the dystrophic changes occurring within mature melanocytes in grey follicles (Orfano, Ruska & Mahrle, 1970; Arck et al., 2006; Nordlund et al., 2008; Tobin, 2009), the causes for greying initiation in humans must be sought within the HFPU, and possibly intra-bulbar melanocyte progenitor cells, but not the bulge. It is also important to note that progressive or temporary loss of melanin transfer from HFPU melanocytes into hair shaft keratinocytes (trichocytes) occurs exclusively during anagen because the functional HFPU exists only during anagen III–VI (Fig. 1) (Tobin et al., 1998).

III. ARE GREYING AND PIGMENTATION GENETICALLY CONTROLLED?

Given the considerable phenotypic overlap between mouse mutants with a hair pigmentation phenotype (Nakamura et al., 2013) and the hair pigmentation abnormalities seen in human patients with corresponding genetic abnormalities, at least some shared essential genes in melanogenesis and melanocyte differentiation, such as tyrosine protein kinase kit (c-kit), MITF, tyrosinase-related proteinase 1 (TRP-1), TRP-2/DCT, paired box gene 3 (PAX3), SRY-box 10 (SOX10), biogenesis of lysosome-related organelles complex 3 (BLOC-3), and neurofibromin 1 (NFI), have been identified (Nordlund et al., 2008; Pingault et al., 2010; Slciman et al., 2013; Feng, Sun & Wang, 2014; Kubasch & Biological Reviews 96 (2021) 107–128 © 2020 The Authors. Biological Reviews published by John Wiley & Sons Ltd on behalf of Cambridge Philosophical Society.
Meurer, 2017; Saleem, 2019). The role of genes in human greying remains poorly understood, but there are clear trends in greying onset within kinships and between populations. The age of greying onset is linked to geographic ancestry, and significant greying is considered premature when it occurs before 20 years of age in Caucasians, before 25 years in populations of Asian ancestry and before 30 years of age in populations with African ancestry (Tobin, 2009; Sonthalia, Priya & Tobin, 2017; Kumar, Shamim & Nagaraju, 2018). Men also grey faster than women, but in distinct scalp regions (Panhard, Lozano & Loussouarn, 2012).

Although ethnic and sex differences suggest a genetic component in the age of greying onset, heritability itself seems to vary regionally; in a twin-controlled study of heritability within Danish and British Caucasians, the onset of greying was highly heritable (Gunn et al., 2009). By contrast, in another larger study of facial and scalp hair features across an ethnically diverse Latin American cohort, hair greying had the lowest heritability of all traits studied (Adhikari et al., 2016), with greying-associated single nucleotide polymorphisms (SNPs) explaining only 6.7% of the observed phenotypic variation. A comparison of individual HFs on the same scalp – which consequently share the same genetic makeup and exposure – indicates a high level of HF-to-HF heterogeneity, and typically early-greying HFs undergo depigmentation decades before other HFs on the same head (Panhard, Lozano & Loussouarn, 2012). This is perhaps most evident in so-called steel/salt and pepper-headed individuals, which can remain a life-long feature. Intra-individual and inter-follicular heterogeneity strongly argues against a solely genetic pathobiology of greying, as it necessarily implicates modifiable factors that operate at the single-HF organ level.

So far, only a single SNP has been significantly associated with greying in an admixed Latin American population with European ancestry. The SNP is in the interferon regulatory factor 4 gene (IRF4) (Adhikari et al., 2016), and mechanistically would be expected to affect the HFPU directly. Since IRF4 and MITF act cooperatively to activate transcription of tyrosinase (TYR) in cultured human melanoma cells (Praetorius et al., 2013), this presumptive driver of human greying (Adhikari et al., 2016) would be associated with reduced tyrosinase activity in the HFPU, rather than in MSC maintenance, since the bulge is constitutively tyrosinase-negative. However, functional evidence that IRF4 really plays an important role in human hair greying is still missing. In a separate study, an HFPU-centric model of greying onset is also suggested by changing patterns of expression of melanogenesis genes during premature greying. In a small sample population, reduced expression of HFPU-resident melanogenic enzymes was accompanied by an increase in their corresponding, complementary inhibitory microRNAs (Bian et al., 2019). Overall, whilst loss of HF niche-resident MSCs is very likely responsible for the irreversibility of hair greying, the process invariably requires and begins with primary changes in melanogenesis, melanosome transfer and/or HF melanocyte survival within the anagen HFPU.

As critical as MITF and tyrosinase are for the control of melanocyte function and melanogenesis (Levy, Khaled & Fisher, 2006; Ganesan et al., 2008; Vachtenheim & Borovanský, 2010; Chen et al., 2018), neither the human nor mouse HFPU appear to be controlled by a single master gene. Instead, the importance of metabolites, co-factors, pH and other biochemical determinants of the local activity of melanogenesis enzymes, as well as reactive oxygen species (ROS) production and scavenging (Schallreuter et al., 1994; Wood et al., 2004, 2009; Slominski et al., 2005b) cannot be overemphasized. Furthermore, the HFPU is subject to a flux of numerous regulatory inputs that jointly ensure its appropriate function, mediating hair cycle-associated changes in HFPU activity and modulating melanin production, melanocyte survival, migration, and proliferation/apoptosis ratios. Currently recognized regulators of the human HFPU are summarized in Fig. 3.

How exactly the expression/activity of these regulators is altered in greying human HFs compared to their fully pigmented counterparts in vivo remains unknown, but ex vivo studies on micro-dissected and organ-cultured human scalp HFs are revealing how these regulators may influence HFPU activity in vivo. Additionally, given the general role of autophagy in skin ageing (Eckhart, Tschachler & Gruber, 2019), that a certain level of autophagic flux in the anagen hair matrix is required for anagen maintenance of human scalp HFs ex vivo (Parodi et al., 2018), and that functional autophagy appears crucial for melanocytes to cope with cellular stress and oxidative damage (Zhou et al., 2018; Kim et al., 2019; Qiao et al., 2020), it is conceivable that insufficient autophagic flux within the HFPU may contribute to hair greying.

Both thyroid hormones [triiodothyronine (T3) and tetraiodothyronine (T4)] (Van Beek et al., 2008) and the central neuroendocrine regulator of the hypothalamic-pituitary-thyroid (HPT) axis, thyrotropin-releasing hormone (TRH), which is produced within human scalp HFs (Slominski et al., 2002b; Gáspár et al., 2010), stimulate melanin production in the HFPU of human anagen VI HFs (Gáspár et al., 2011). Pigmentation is also regulated by the activity of an intrinsic hypothalamus–pituitary–adrenal (HPA) axis within the follicle, such that upstream corticotropin-releasing hormone (CRH), its receptor, as well as downstream adrenocorticotropic hormone (ACTH), alpha melanocyte-stimulating hormone (α-MSH), and the melanocortin receptors are all produced within the HF itself and promote pigmentation (Slominski et al., 1999, 2004a, 2004b; Ito et al., 2005; Kauser et al., 2005, 2006; Van Beek et al., 2008; Meyer et al., 2009; Gáspár et al., 2011). Study of hair pigmentation in knockout mice unable to synthesize downstream HPA hormones suggests that their action is secondary to TRH, which promotes pigmentation in their absence (Slominski et al., 2005a) likely via the melanocortin receptor (Schöth et al., 1999; Slominski et al., 2002b), although this is yet to be verified in the human HF. Whilst known aspects of neuroendocrine regulation of hair pigmentation have been reviewed in detail elsewhere (Slominski et al., 2004b; Paus et al., 2014), the extent to which changes in these hormonal axes intrinsic to the HF affect the greying process has not been systematically investigated. This also
applies to cytokines and growth factors such as nerve growth factor (NGF), stem cell factor (SCF) and hepatocyte growth factor (HGF) that protect human HFs from pigment loss **ex vivo** (Botchkareva et al., 2001; Campiche et al., 2019).

Hormonal stimulation of human melanocytes by the intrinsic HPA-like axis (Price et al., 1998), or by TRH (Gáspár et al., 2011) promotes MITF expression and it is likely that reduced intrafollicular production of and stimulation by melanotropic hormones may result in lowered MITF activity within the HFPU of greying human HFs, ultimately causing insufficient melanogenesis and melanosome transfer (Slominski et al., 2004b; Paus, 2011). As illustrated in Fig. 4, a reduction of these melanotropic HPA hormones can be observed in the hair bulb epithelium of grey/white human scalp HFs, while hair pigmentation-stimulatory drugs such as fluoxetine have been suggested to up-regulate intrafollicular α-MSH expression in some white human scalp HFs **ex vivo** (Chéret et al., 2020). This provides further circumstantial support for the concept that a relative decline in the intrafollicular production of key melanotropic neurohormones contributes to the greying process (Paus, 2011; Paus et al., 2014).

A fundamentally important concept is that HF cycling controls HFPU activity, and thereby hair greying, insofar as defective hair shaft pigmentation can only occur during anagen (there is currently no evidence that isolated it changes in HFPU activity can regulate HF cycling). It therefore deserves emphasis that some recently discovered, potent intrafollicular regulators of human HF cycling also regulate the HFPU and its melanin production in a hair cycle-independent manner, namely P-cadherin (Samuelov et al., 2012, 2013), TRH (Gáspár et al., 2010, 2011), and peripheral clock activity [i.e. circadian locomotor output cycles kaput (CLOCK), brain and muscle ARNT-like 1 (BMAL1)] (Al-Nuaimi et al., 2014; Hardman et al., 2015). The roles of such dual regulators that independently impact upon human anagen duration and intrafollicular melanin production have not been systematically dissected in the context of greying.

**IV. THE ROLE OF MELANOCYTE STEM CELLS IN GREYING**

To guide future efforts in human hair greying research, it is important to avoid conceptual confusion. Ever since the
landmark discovery of bulge MSCs and their involvement in hair greying (Nishimura et al., 2002; Nishimura, Granter & Fisher, 2005), it has become a frequently repeated misconception that damage to bulge MSCs is the primary cause of human hair greying (e.g. Jo et al., 2018; Qiu et al., 2019). As explained above, greying of human scalp HF begins within the highly differentiated melanocytes of the HFPU within a single anagen phase. As there is no evidence to support the view that continuous replenishment of the scalp HFPU from the bulge MSC reservoir is required to support a single anagen VI phase in human HFs, the latter appears to be self-maintained.

However, the verdict that greying always results initially from major HFPU dysfunction in the anagen hair matrix, i.e. far distant from the bulge and independent of MSC activities, does not preclude the simultaneous, independent accumulation of (eventually irreversible) MSC damage over time in the bulge stem cell niche. As there is no evidence to support the view that continuous replenishment of the scalp HFPU from the bulge MSC reservoir is required to support a single anagen VI phase in human HFs, the latter appears to be self-maintained.

In mice, MSC progeny from the sub-bulge region repopulate the secondary hair germ before the onset of each new anagen stage (Nishimura et al., 2002). Histological snapshots from the hair cycle indicate a homologous series of events in humans, although it remains ambiguous which MSC sub-populations exactly contribute to the rebuilding of the human HFPU during anagen development (Commo & Bernard, 2000). Failure to maintain this stem cell niche is associated with a variety of different greying phenotypes in mouse models, with some translatable insights into humans. For instance, mice deficient in B-cell lymphoma 2 (Bcl2), an anti-apoptosis factor that preserves mitochondrial membrane integrity (Kale, Osterlund & Andrews, 2018), exhibit rapid greying at 8 weeks of age, as all MSCs are lost from the niche after the first anagen phase (Nishimura, Granter & Fisher, 2005). Reportedly, loss of MSCs in these mice was not due to apoptosis, but rather resulted from their irreversible 'ectopic differentiation' into pigmented, dendritic melanocytes. Likewise, evidence of ectopic differentiation within MSCs was reported in humans during middle age (Nishimura, Granter & Fisher, 2005), but not in senile white HFs (Commo, Gaillard & Bernard, 2004; Nishimura, Granter & Fisher, 2005). Therefore, understanding the molecular machinery that preserves MSC stemness and quiescence (Fig. 5) remains an important facet of understanding the entire greying process.

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Stress-induced sympathetic nervous activity has been implicated in driving ectopic MSC differentiation in mice (Zhang et al., 2020). Along with radiation-induced poliosis (Gao et al., 2019), these experimental tools must not be equated and conflated with spontaneous, physiological ageing-associated greying in human HF. However, it is instructive to highlight that increased exposure to ionizing radiation and genotoxic reagents during telogen results in increased levels of ectopically differentiated MSCs and poliosis during the next anagen phase (Inomata et al., 2009). Additionally, compromised signal transduction upstream of DNA repair mechanisms lowers the dose of ionizing radiation required to produce both ectopic pigmentation in the bulge and a grey phenotype (Inomata et al., 2009). It is noteworthy that reactive oxygen species (ROS) are also well-known inducers of DNA damage and inhibit DNA repair (Van Houten, Santa-Gonzalez & Camargo, 2018). The effects of oxidative damage upon MSC maintenance in the human HF remains poorly understood, but this should constitute a key component of further efforts to understand the progression of greying in humans (Jo et al., 2018).

Similarly to ageing, hypomorphic mutation of Mitf elicits a more gradual process of greying (Lerner et al., 1986) associated with progressive ectopic differentiation of MSCs in the sub-bulge region of unpigmented hairs (Nishimura, Granter & Fisher, 2005; Harris et al., 2018). Current evidence in mice suggests that bulge-resident epithelial HF stem cells (eHFSCs) maintain adjacent MSCs in an immature state with quiescence-promoting transforming growth factor beta (TGF-β) signalling that is dependent on the expression of eHFSC-derived collagen alpha-1(XVII) chain (Col17a1) (Nishimura et al., 2010; Tanimura et al., 2011) (Fig. 5). Genotoxic damage results in proteolysis of Col17a1 and ectopic differentiation of MSCs (Matsumura et al., 2016). Knocking out either Col17a1 or TGF-β in mice induces gradual greying and accompanying ectopic differentiation of MSCs in the sub-bulge. When MSCs are required to populate the hair germ during telogen, Wingless-related integration site (Wnt) signalling and endothelin originating from eHFSCs are subsequently required for MSC proliferation and differentiation (Rabbani et al., 2011), and are therefore candidate signals in the acceleration of greying. It remains unknown to what extent these controls of quiescence and differentiation (Fig. 5) are mirrored in the maintenance of human MSCs in the bulge and other HF compartments. Characterising these surely is important to mechanistically understand the progression of greying to an irreversible stage.

V. THE ROLE OF OXIDATIVE STRESS IN GREYING

While a key role for oxidative stress in greying has long been appreciated (Trüeb & Tobin 2010, Paus 2011, Arck et al. 2006, Wood et al., 2009), many details on how exactly excessive ROS levels damage the HFPU are as yet insufficiently understood.

(1) Management of oxidative stress within the HF

Within melanocytes, tyrosine hydroxylation and DOPA-to-melanin oxidation in the melanogenesis pathway lead to high levels of ROS release, which are managed by an efficient local antioxidant system (Paus, 2011; Trüeb, 2015). This system include catalase, methionine sulfoxide reductase A and B (MSRA/MSRB) (Wood et al., 2009), Bcl-2 (Peters, Arck & Paus, 2006), nuclear factor erythroid 2-related factor 2 (Nrf2) (Haslam et al., 2017), tyrosinase-related protein-2 (TRP-2) (Michard et al., 2008), and even intrafollicular melanin production (Kobayashi et al., 2005; Fischer et al., 2008). Eumelanin itself also effectively scavenges ROS (Wood et al., 2011).

![Fig 5. Melanocyte stem cell (MSC) depletion contributes to hair shaft greying in mice. (A) During telogen, MSCs in the sub-bulge niche are required to migrate to the secondary hair germ in order to replenish the hair follicle pigmented unit (HFPU). Similarly to humans (Tobin et al., 1998). (B) MSCs must be maintained in a quiescent state between episodes of replenishment. Transforming growth factor beta (TGF-β) is an important signal emitted from the surrounding epithelial hair follicle stem cells (eHFSCs) to promote quiescence. (C) Failure to maintain MSC quiescence due to ageing, genotoxic stress and adrenergic activity results in ectopic MSC differentiation within the niche. Differentiated melanocytes are no longer able to replenish the HFPU, and greying becomes permanent. Ectopic pigmentation within the bulge has also been reported during human hair follicle greying (Commo, Gaillard & Bernard, 2004; Nishimura, Granter & Fisher, 2005). Col17a1, collagen alpha-1(XVII) chain; SNS, sympathetic nervous system.]
et al., 1999), although its antioxidant activity is sequestered within and restricted to the melanosome itself. Thus, it has been proposed that impaired antioxidant systems and over-accumulation of ROS cause melanocyte damage during ageing, with external stimuli [inflammation, ultraviolet (UV), smoking and oxidizing agents] also contributing to the loss of redox balance (Tobin & Paus, 2001) (Fig. 6).

The HFPU is sensitive to oxidative damage, as can be seen by exposing HFs to H$_2$O$_2$ (Arck et al., 2006, Wood et al. 2009) or ROS-generating cytotoxic agents that induce lipid peroxidation, mitochondrial DNA (mtDNA) deletion and cell death (Bodó et al., 2007; Haslam et al., 2017). Greying hair bulbs often display vacuolated melanocytes, a common cellular response to oxidative stress (Nordlund et al., 2008; Tobin, 2009). A recent study reported specific nuclear expression of ataxia-telangiectasia mutated (ATM; a serine/threonine protein kinase that is a major regulator of the cellular response to DNA double-strand breaks) within the HFPU. ATM expression increases in primary human scalp HF melanocytes in response to incubation with H$_2$O$_2$ and was essential for maintaining melanocyte viability during oxidative stress. Expression of ATM correlated positively with the pigmentary status of greying HFs (Sikkink et al., in press), suggesting that reduced capacity of melanocytes to resist genotoxic challenge is also characteristic of the greying follicle.

Fig 6. Oxidative stress plays a key role in hair greying. (A) In the bulge region of grey hair follicles (HFs), human melanocyte stem cells (MSCs) are reduced in number and undergo ectopic differentiation. The hair shaft displays millimolar concentrations of H$_2$O$_2$ and oxidation of methionine residues to methionine sulfoxide (Wood et al., 2009). (B) In pigmented HFs, the pre-cortical zone displays the ‘ring-of-fire’, an area containing high amounts of reactive oxygen species (ROS), suspected to be crucial in keratinocyte (KC) differentiation and hair shaft (HS) formation (Lemasters et al., 2017), whereas the HF bulb contains lower ROS levels. In grey HFs, an increase in ROS correlates with decreased melanocyte number, increased melanocyte death and increased mitochondrial DNA (mtDNA) deletion (a marker of accumulating oxidative stress damage) (Arck et al., 2006). Grey HFs exhibit a reduction in the antioxidant enzymes catalase and methionine sulfoxide reductase A and B (MSRA/MSRB) (Wood et al., 2009; Shi et al., 2014). It is also possible that grey HFs exhibit an accumulation of senescent melanocytes that cannot be removed through apoptosis. (C) The roles of melatonin in human HF pigmentation, both proven and hypothetical (marked ‘?’). BMAL1, brain and muscle ARNT-like 1; CLOCK, circadian locomotor output cycles kaput; Nrf2, nuclear factor erythroid 2-related factor 2.
There is emerging evidence that human HF melanocytes, compared to epidermal melanocytes, are more susceptible to chronological ageing, as evidenced by their unique progressive loss of catalase expression and activity (Kauser et al., 2011). A comprehensive characterization of changes in prematurely grey HFs compared to pigmented ones shows downregulated gene expression of the pigmentation-associated genes TTR, DCT, TTRP1, melanocortin 1 receptor (MC1R) and MITF, as well as downregulation of CAT (catalase), GPX1 (glutathione peroxidase 1) and SOD (superoxide dismutase) (Shi et al., 2014), suggesting that the collapse of the antioxidant system within HFPU melanocytes plays a critical role in greying initiation. Grey and white human HFs also show reduced levels of MSRA/MSRB (Wood et al., 2009). Additionally, the roles of glutathione-s-transferases (GSTs), a class of enzymes with antioxidant properties that offer protection against oxidative stress (Da Fonseca et al., 2010), should also be studied in the context of human hair greying since human HFs express GSTs (Dijkstra et al., 1996; Puerto et al., 1994); GST levels reportedly decrease by 78% with age alongside an 88% drop in glutathione reductase (Pruche, Kermici & Prunieras, 1991), suggesting a compromised capacity to protect against peroxides as HF ageing progresses.

Interestingly, vasoactive intestinal peptide (VIP) receptor expression is detected in the murine bulge during anagen IV (Wollina, Paus & Feldtrappe, 1995). In human anagen HFs, VIP receptors vasoactive intestinal peptide receptor 1 (VPAC1) and VPAC2 are also expressed, and are reduced in alopecia areata (Bertolini et al., 2016b), raising the question whether VIP signalling is also relevant in the context of hair greying and oxidative stress, since VIP has been shown to counteract ROS production through inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and stimulation of SOD2 (Fujimori et al., 2011). Treating human HFs with VIP increases the number of c-Kit+ HF melanocytes (most of which are immature/amelanotic), gp100+, MITF+ and phospho-MITF+ HFPU melanocytes, as well as melanin synthesis; melanogenesis is also stimulated during in vitro culture of isolated HFPU melanocytes following VIP treatment (Shi et al., 2014; Bertolini et al., 2016a).

It has also been shown that methionine residue 374 (Met374) of tyrosinase, the rate-limiting enzyme of melanogenesis, constitutes a prime target for H$_2$O$_2$-mediated oxidation (Wood et al., 2009). Thus, insufficiently scavenged H$_2$O$_2$ within the HFPU could promote oxidation of methionine sulfoxide (Met-S=O), resulting in a dose-dependent inhibition of tyrosinase activity and so of HF melanogenesis which is preventable by free L-methionine application (Wood et al., 2009) (Fig. 6). Accumulation of excessive H$_2$O$_2$ in the presence of reduced catalase activity could therefore directly damage the HFPU’s enzymatic melanin synthesis and its capacity to protect itself from oxidative damage by melanin production, resulting in a vicious circle.

(2) Sources of ROS in and around the HFPU

Oxidative damage of the HFPU can result from failure of the antioxidant control systems described above, but alternatively has been attributed to excessive intrinsic and extrinsic ROS production (Fig. 6). Melanogenesis itself generates substantial amounts of superoxide radical (Riley, 1988) and H$_2$O$_2$ (Koga, Nakano & Tero-Kubota, 1992; Nappi & Vass, 1996), and therefore represents a major source of melanocyte-intrinsic oxidative stress. If the ROS-generating enzymatic cascade culminating in melanin synthesis is stimulated, but the ROS-scavenging end-product eumelanin is insufficiently synthesized, e.g. owing to an inhibition of post-DOPA steps of melanogenesis (Slominski, Paus & Bomirski, 1989; Slominski et al., 2005b), or blunting of redox balancing capacity, this could result in major oxidative damage to the HFPU.

Within the proximal HF ORS and hair matrix, human HF keratinocytes are richly endowed with energetically active mitochondria (Vidali et al., 2014), and when HF keratinocytes terminally differentiate in the IRS and pre-cortical hair matrix, they show heightened membrane potential and ROS generation, after which they abruptly depolarize and secrete a circumferential spike of ROS, described as the HF ‘ring of fire’ (Lemasters et al., 2017). Thus, insufficient scavenging controls of this region would be expected ultimately to damage the HFPU. Co-culturing human neonatal epidermal melanocytes with epidermal keratinocytes in which catalase is inhibited increases ROS levels within the melanocytes (Pelle et al., 2005), suggesting that compromised antioxidant systems in neighbouring keratinocytes may indeed contribute to melanocyte destruction or impairment.

It has been hypothesized that ‘bleaching’ of the melanin polymer itself by diffusible H$_2$O$_2$ may be responsible for the striking phenomenon of sudden greying (canities subita) (Paus, 2011). However no ‘bleached’ melanosomes have yet been identified in the hair shaft ultrastructure of typical grey/white HFs. Instead, initially, the grey hair shaft seems to be characterized by reduced density of smaller, yet still pigmented melanosomes (Cho et al., 2014). Additional melanocyte-extrinsic modulators of ROS are human perifollicular mast cells (Jadkaukaite et al., 2018) and Substance P-mediated neurogenic inflammation (Peters et al., 2007). In human HFs, Substance P activates mast cell degranulation and induces catagen, thus turning off melanogenesis. Like the HF (Haslam et al., 2017), human perifollicular mast cells also show substantial NRF2 activity, which can counteract oxidative damage associated with Substance P-induced mast cell degranulation (Jadkaukaite et al., 2018).

Lastly, UV radiation may also constitute a major and extrinsic source of ROS for HFs (Zhang et al., 1997; De Jager, Cockrell & Du Plessis, 2017; Slominski et al., 2018). When depilated mice were irradiated with UV-A after applications of a gel containing the light-sensitive drug psoralen, and the antioxidant enzyme SOD, black hair re-grew on their backs; however, when no SOD, or heat-inactivated SOD was added together with the psoralen, solely grey or white hairs regenerated during anagen in 90% of treated mice (Emerit et al., 2007). Therefore, topical application of SOD, presumably penetrating via the transfollicular route, acted to prevent HFPU melanocyte-specific damage in these mice. It
remains unclear whether MSCs were also affected in this study, or whether greying was reversible long term.

Irradiating human HF s \textit{ex vivo} with 20 or 50 mJ/cm² UV-B radiation impaired hair shaft elongation, HFPU melanogenesis and induced premature catagen via both apoptotic cell death (20 mJ) and necrosis (50 mJ) (Lu et al., 2009). These human HF s did not undergo greying similar to the murine study (Emerit et al., 2007), and both keratinocytes and melanocytes were affected similarly. This is likely owing to the lack of epidermis, dermis and dermal white adipose tissue within the experimental design, the former of which is biologically designed to absorb a high amount of UV radiation with specific antioxidant mechanisms acting as protective agents (Brand et al., 2018; Dunaway et al., 2018). However, UV radiation may have a more damaging role to the HFPU than previously thought, since recent evidence suggests that transepidermally applied UV irradiation exerts substantial HF photodamage, which can be counteracted by topical caffeine \textit{ex vivo} (Gherardini et al., 2019).

VI. MAJOR OPEN QUESTIONS IN HUMAN HAIR GREYING AND HOW TO ANSWER THEM

Many open questions that remain to be addressed regarding the gerontobiology of human HF pigmentation have already been listed above. In the following paragraphs, these are extended to illustrate why this skin research frontier provides such a treasure trove of interdisciplinary biomedical investigation at the intersection of ageing/senescence research, pigmentation, stem cell, and oxidative damage biology.

(1) Can changes in the pigmentary status of individual follicles be tracked over time, and related to trends in the physiology of ageing?

A desirable approach to studying the biology of greying would be to look critically at subjects over a period of months to years, tracking a selected population of HF s to measure changes in hair fibre melanin both optically and via biophysical methods, e.g. electron paramagnetic resonance spectroscopy (Plonka, Plonka & Paus, 1995; Slominski et al., 1996). In this way, changes in pigmentary status may be correlated with other physiological and/or environmental variables. The development of computational models to relate hair growth rate to changes in hair shaft pigmentation would also help greatly in tracking the timing of de- and re-pigmentation events within single follicles, thus allowing historical analysis of relevant stressors or physiological changes reported by a subject.

(2) How can human MSCs be reliably identified?

Attempts to identify early-stage greying-associated changes in these cells require reliable, sensitive and selective methods/markers. Yet, no single definitive and highly specific \textit{bona fide} MSC marker has been convincingly demonstrated to date (Osawa et al., 2005; Harris et al., 2013; Li & Hou, 2018), even in the mouse. Whilst some pragmatic consensus has been reached for murine MSC markers (Joshi et al., 2019), no currently available positive or negative read-out parameters [e.g. c-Kit, cluster of differentiation 34 (CD34), DCT, gp100+/CRH+, SOX10] provide definitive proof that a putative human MSC is being labelled. Moreover, what exactly defines a MSC is a matter of debate, since its melanocyte colony-forming capacity is neither reliable nor distinctive, as neighbouring stem/progenitor cells, such as nestin+ cells and some Schwann cells, also possess this capacity (Dupin et al., 2003; Amoh et al., 2005; Tieche et al., 2009a; Li et al., 2010; Van Raamsdonk & Deo, 2013); even human CD34+ cells may differentiate into melanocytes (Trempus et al., 2003; Ito et al., 2004a).

All published claims regarding so-called ‘MSCs’ must therefore be interpreted with caution: were genuine (and all) MSCs and specific sub-populations labelled, analysed and manipulated? And which ones were missed or lost during experimentation? This major handicap to murine and human MSC-related studies is rarely acknowledged even in high-impact publications and hampers research into the role of MSCs in human hair greying. Therefore, this lack of availability of selective \textit{in vivo}-labelling and imaging methods for native MSCs in human skin remains a significant challenge.

(3) Do CRHR+ amelanotic melanocytes in the proximal hair bulb represent an alternative MSC pool?

The most proximal region of the HF bulb contains a population of CRH receptor positive (CRHR+) amelanotic melanocytes (Ito et al., 2004b), whose characteristics and function remain unknown. The hypothetical role of CRHR+ amelanotic melanocytes as HFPU-replenishing MSCs proposed here is important for several reasons. The spatial closeness of these cells to the HFPU compared to the MSCs in the bulge raises the possibility that they could continuously replenish the mature melanocytes during the many years of a single scalp HF anagen, without any need for recruitment from bulge MSCs. Moreover, during catagen, these CRHR+ putative MSCs may retreat into the secondary hair germ and reconstruct a new HFPU during the subsequent anagen phase. If this is the case, these cells likely play an integral role in the human greying process. Their loss, for instance due to oxidative stress, would result in insufficient maintenance of melanocyte density and melanogenesis in the HFPU during anagen. Activation and differentiation of these cells would also offer an explanation for the intriguing phenomenon that even grey HFs can undergo repigmentation \textit{in vivo} (see Table 1) (Chéret et al., 2014) and \textit{ex vivo} (e.g. Chéret et al., 2020) within a single anagen phase.

(4) How do MSC functions change before, during, or after greying onset in the human HFPU?

The chronology of greying-associated MSC changes in relevant HF compartments (Figs 1 and 2) before, during
Table 1. The reversibility of greying: Reported human hair follicle re-pigmentation phenomena (examples)

| Medication class or disease | Medication | Effects | Reference(s) |
|-----------------------------|------------|---------|--------------|
| Psychoemotional stress-induced | n/a | Temporary greying reported to be reversed following the end of intense neurological episodes (epilepsy, seizures, psychosis) | Reviewed in Nahm, Navarini & Kelly (2013) |
| Spontaneous repigmentation | n/a | Hair repigmented in a patient with premature greying | Tobin & Cargnello (1993) |
| Porphyria cutanea tarda | n/a | Repigmentation of previously grey HFs in two female patients (69 and 71 years old), along with facial hypertrichosis | Shaffrali, Mcdonagh & Messenger (2002) |
| Paraneoplastic hypertrichosis | n/a | Darkening of skin and hair in patients with ACTH-induced lung cancer | Karakas et al. (2016); Sabir, James & Schuchter (1999); Tas (2018) |
| Anti-depressant (serotonin re-uptake inhibitor) | Fluoxetine | Human anagen VI HFs show increased pigmentation following ex vivo treatment with Fluoxetine | Chéret et al. (2020) |
| Hyperthyroidism | L-thyroxine | Hair repigmentation reported with excessive endogenous T4 levels or T4 medication; treating human anagen HFs with thyroid hormones T3 and T4 also enhances intrafollicular melanin synthesis ex vivo | Langheinrich et al. (2004); Van Beck et al. (2013) |
| Discontinuation of chemotherapy | n/a | Melanocyte repopulation within the HFPU occurs in some patients after cessation of chemotherapy; described as repigmented hair shafts with white tips of newly grown hair shafts | Rossi et al. (2019); Pirmez, Piñeiro-Maceira & Sodré (2013); Haslam & Smart (2019) Paus et al. (2013) |
| Tyrosine kinase Inhibitors | Erlotinib | Gradual HF repigmentation of hair 3 months to 2 years after treatment initiation | Alexandrescu, Kauffman & Dasanu (2009); Cheng, Chen & Chiu (2014) |
| Sorafenib | When hair regrowth occurs, HFs are occasionally more pigmented than prior to start of treatment | Robert et al. (2009) |
| Other drugs | Tamoxifen | Hyperpigmentation 2.5 years later | Hampson et al. (1995) |
| Cisplatinum | 16% patients (15–54 years of age) showed hair regrowth and repigmentation | Ricci et al. (2006) |
| Lenalidomide | Treatment for myeloma results in gradual hair repigmentation | Dasanu, Misis & Alexandrescu (2013) |
| Thalidomide | 3 years after start of treatment, patients display darkened scalp hair | Lovering et al. (2016) |
| Parkinson’s disease | Levodopa | Diffuse hair repigmentation 8–9 months following treatment initiation | Grainger (1973); Robertson, Faulkner & Vernon (1982) |
| Glaucoma | Latanoprost eye drops | Scattered HF repigmentation 3 years later | Bellandi et al. (2011) |
| Lentigo maligna | Imiquimod | Pigmentation reversal of hyperpigmented HFs undergoing an 8-year anagen phase | Lackey et al. (2019) |
| Anti-inflammatory /immunomodulatory | INFα for Hepatitis C | 2 months following treatment start caused hair repigmentation which persisted after administration was discontinued | Pavithran (1986) |
| Acrinetin, Etretinate | These vitamin A derivatives cause sporadic repigmentation of grey hair | Vesper & Fenske (1996); Seckin & Yıldız (2009); Ward, Miller & Shipman (2014); Nagase, Inoue & Narisawa (2017) |
| 8-methoxy-psoralen (psoriasis) | 46% of patients aged 12–20 treated with PUVA for 13 months showed complete scalp re-pigmentation with no relapse 8 months after | Pavithran (1986) |
| Secukinumab (psoriasis) | Hyperpigmentation of white hair following 6 months of therapy | Rongioletti, Mugheddu & Murgia (2018) |
| Prednisone | Almost-complete repigmentation following 4 months of corticosteroid treatment | Khaled et al. (2008) |
| Cyclosporine | Hypertrychosis and hair darkening occurred 2 months after start of treatment | Rebora, Delmonte & Parodi (1999); Sharma, Dobry & Linden (2019) |
| Adalimulab | Inhibiting TNFα (a melanogenesis suppressor) using Adalimulab induces hair repigmentation | Tintle et al. (2015) |
| Lepromatous leprosy | Clofazimine | | Philip, Samson & Simi (2012) |
and after the onset of declining human HFPU function as well as their temporal association with the hair cycle remain to be systematically defined. Functional assays (e.g. the melanocyte-generating potential in vitro or ex vivo), laser-capture microdissection-based approaches (Ohyama et al., 2006; Tiede et al., 2009b; Harries et al., 2013) and single-cell transcriptional profiling (Cheng et al., 2018; Devitt et al., 2019; Joost et al., 2020) of these specific HF compartments, namely in younger individuals early during the onset of greying, appear best suited to characterize these changes. Such analyses should clarify: (i) which HF compartment is functionally most relevant to the biology of human hair greying (i.e. bulge versus most proximal hair matrix) (Figs 1, 2 and 6); (ii) which key genes and signalling pathways of human melanocyte biology and melanogenesis change first; and (iii) the temporal sequence in which this occurs in relation to other HF compartments. This approach should also make it possible to correlate MSC-related changes with greying-associated alterations in directly adjacent epithelial progenitor cells.

(5) Which functional and epigenetic changes occur within HFPU melanocytes and MSCs during human HF ageing?

This approach should be complemented with a systematic search for functional changes in a hypothesis-driven manner via in situ analyses. For example, how do autophagy, MITF expression/activity, mitochondrial activity, metabolic profile, telomere length/function, DNA damage/stress sensing and other senescence markers including senescence-associated secretory profile (SASP) molecules and/or molecular indicators of oxidative DNA and cell membrane damage change before human HF melanogenesis visibly declines? An additional layer of complexity is epigenetic changes that remain completely unknown in the context of human hair greying, including DNA and histone methylation, histone acetylation and chromatin remodelling (Orioli & Dellambra, 2018).

(6) How does the niche environment of key greying-related human HF compartments change?

Recent studies have identified a key role of the bulge extracellular matrix composition for stem cell function and ageing (Matsumura et al., 2016). This complements previous insights into the importance of P-cadherin expression in the most proximal human HM (Samuelov et al., 2013), and the key role of E-cadherin for human melanocyte biology (Wagner et al., 2015; Keswell, Kidson & Davids, 2015; Singh et al., 2017). Hence, it appears necessary to search for distinct changes in the extracellular matrix- and adhesion molecule-related niche environment before greying onset as well as during its stepwise progression. This should be correlated with greying-associated changes in the local expression/activity of key growth factors and neurohormones (e.g. SCF, HGF, CRH, α-MSH, β-endorphin, TRH). Since glycobiological changes, such as ageing-associated accumulation of advanced non-enzymatic glycosylation end-products, promote cell and tissue ageing (e.g. Sinn et al., 2015; Cardoso et al., 2018), these analyses ideally should be complemented by assessing ageing-associated changes in protein glycosylation.

(7) Is the human HFPU replenished during anagen, and – if so – by which progenitors?

If human MSCs do not replenish the HF bulb during a single anagen phase (multi-year on the scalp), greying initiation
must be entirely controlled within the HF bulb. This scenario would impact on the design of the most sensible anti-greying therapeutic strategies (see Section VII). The same applies if bulge MSCs or their immediate progeny are indeed required continuously to replenish the human HFPU and whether this replenishment process becomes defective before the onset of greying.

Unfortunately, only a short window of the anagen VI–catagen and telogen–early anagen transformation can be studied in human HF organ culture (Langan et al., 2015; Hawkshaw et al., 2019), and lineage tracing is extremely challenging with currently available human HF organ culture techniques. Therefore, xenotransplantation of human scalp HFs onto severe combined immunodeficient (SCID) mice (Gilhar et al., 2016; Oh et al., 2016) should facilitate the long-term tracking of MSC and melanocyte dynamics in vivo. However, this requires the identification of a promoter that is exclusively active in human MSCs, which would permit, for example, GFP labelling (as has been accomplished for human epithelial HF stem cells via the keratin 15 promoter (Tiede et al., 2009); R a m o t et al., 2017) of mitochondrial motility and fusion/fission dynamics following oxidative stress using MitoCLox (Lyamzaev et al., 2020); mitDNA copy number (copies of mitochondrial genome per cell) (Malik et al., 2011); oxidative damage marker 8-hydroxy-2’-deoxyguanosine (8-OHdG) (Gherardini et al., 2019), and mtDNA mutations/deletions (Bodo et al., 2007; Lu et al., 2009; Picard et al., 2012, Rygiel et al., 2016); and (x) multiphoton imaging of mitochondrial motility and fusion/fission dynamics (Lemasters et al., 2017).

(8) How exactly does oxidative stress and mitochondrial damage impact on the HFPU during human hair greying?

Although multiple studies suggest a major contribution of oxidative stress to the biology of hair greying in mice (Liu et al., 2013) and human HFs (Peters, Arck & Paus, 2006; Wood et al., 2009; Kauser et al., 2011; Haslam et al., 2017; Sikkink et al., in press), the exact downstream mechanisms have not been fully elucidated. For instance, to what extent does oxidative stress damage mtDNA within HFPU melanocytes and promote their senescence? And what is the contribution of mitochondrial dysfunction, altered dynamics and excessive ROS generation (Jang et al., 2018) to HFPU damage during greying? This may be studied in vitro on isolated human HF melanocytes (Tobin, Colen & Bystryn, 1995; Zhu et al., 2004; Kauser et al., 2006). However, these ORS-derived cells initially are amelanotic and do not represent HFPU melanocytes when they switch on melanogenesis in vitro. However, there is evidence that these cells retain their HF identity for at least several passages in vitro (Tobin & Bystryn, 1996; Kauser et al., 2011).

In situ analyses of HF melanocytes and their progenitor cell populations in organ-cultured early-stage greying HFs (Langan et al., 2015) can be used to assess (i) mitochondrial position and morphology through dyes like MitoTracker (Harwig et al., 2018); (ii) mitochondrial membrane potential through TMRM/TMRE and JC1 (Wolf et al., 2019); (iii) mitochondrial enzymatic activity for respiratory chain complexes I, II, III and IV by enzyme histochemistry (Vidali et al., 2014; Simard et al., 2018); (iv) mitophagy [immunostaining for PTEN-induced kinase 1 (PINK1) and PINK2 combined with light chain 3B (LC3B)/sequestosome 1 (SQSTM1)] (Gökerkılıç, Tramier & Bertolin, 2020); (v) mitochondrial fission and fusion, through quantification of major players of mitochondrial fission [mitofusin-1 (MFN1), MFN2 and optic atrophy 1 (OPA1)] and fission/dynamin-related protein 1 (DRP1)] (Miret-Casals et al., 2018); (vi) mitochondrial biogenesis markers like the transcription factor proliferator-activated receptor gamma coactivator 1-alpha [PGC1α] and mitochondrial mass markers porin/voltage-dependent anion-selective channels (VDAC) and translocase of outer mitochondrial membrane 20 (TOMM20) (Vidali et al., 2014; Fu, Liu & Yin, 2019); (vii) lipid peroxidation dynamics following oxidative stress using MitoCLox (Lyamzaev et al., 2020); (viii) mtDNA copy number (copies of mitochondrial genome per cell) (Malik et al., 2011); (ix) oxidative damage marker 8-hydroxy-2’-deoxyguanosine (8-OHdG) (Gherardini et al., 2019), and mtDNA mutations/deletions (Bodo et al., 2007; Lu et al., 2009; Picard et al., 2012, Rygiel et al., 2016); and (x) multiphoton imaging of mitochondrial motility and fusion/fission dynamics (Lemasters et al., 2017).

(9) Is a decline in HF oxidative damage control and repair systems a primary driver of human hair greying?

It remains unknown whether spikes of excessive ROS generation in or around the HFPU initiate human hair greying, or whether primarily a decline in ROS scavenging capacity and oxidative damage response and repair functions are responsible. Perhaps both must coincide to impair HFPU function lastingly and significantly? Determining the molecular changes that occur in greying hairs, by quantifying the hair proteome of dark versus white hairs or post-translational molecular modifications in different segments of the hair shaft, could offer insight into the role of ROS in relation to the temporal aspects of greying dynamics.

Could BCL-2, the mitochondrial anti-apoptotic protein that is down-regulated in human grey HFs (Wood et al., 2009), be a primary target of oxidative stress, resulting in HF melanocyte apoptosis as a mechanism of greying? Alternatively, could ROS be responsible for initiating an autoimmune greying process, as is suspected in vitiligo (Schallreuter et al., 2013)? In addition, H2O2-mediated oxidation has been demonstrated for pigmentation regulators including propionimelanocortin (POMC), a-MSH and b-endorphin (Kauser et al., 2003; Spencer et al., 2008, 2007), as well as synthesis and recycling of 6-tetrahydrobioppterin (Schallreuter et al., 1994). Since these important pigmentation regulators are required for L-phenylalanine-to-L-tyrosine conversion (Schallreuter & Wood, 1999), it is conceivable that they become insufficient/defective at the onset of greying.

Moreover, what exactly induces uncontrolled ROS spikes and/or a decline in oxidative damage repair, and why does this occur three decades after perfect functioning of intrafollicular oxidative stress control in the HF, yet decades before hypopigmentation events are routinely seen in human
epidermis? And what accounts for the obvious vulnerability of the temporal scalp HFs that first undergo greying compared to the occipital HFs of the same scalp that may not undergo greying until 2–3 decades later? Once this has been clarified, it will finally be possible to follow up the concept of dysregulated redox balance within greying HF melanocytes (Tobin & Paus, 2001; Trüeb, 2015) with precisely targeted and coordinated anti-greying interventions.

(10) What can we learn from reversible greying phenomena?

Clinical phenomena of reversible hair greying (Table 1) deserve more attention from biologists, as they provide intriguing pointers to signalling pathways and lead agents that may restore HFPU functionality or its correct replenishment by MSCs. In order to dissect the unknown mechanisms that underlie these greying reversal phenomena, fully pigmented versus grey/white scalp HFs could be exposed in HF organ culture to candidate repigmentation-inducing candidate drugs (Chéret et al., 2020) and hormones. In addition, molecular studies in segments of single hair shafts exhibiting a white-to-dark repigmentation transition could also provide complimentary information about the cellular recalibrations associated with the reversal of greying in human hairs.

VII. THERAPEUTIC IMPLICATIONS AND PERSPECTIVES

What the greying-reversal phenomena (Table 1) robustly document is that human hair greying is reversible, in principle, for longer than is credited. Therefore, the therapeutic window of opportunity during which HF pigmentation decline can be halted, restimulation of melanin synthesis and transfer to keratinocytes can be achieved, and the progression of hair greying towards irreversibility can be slowed or prevented spans the entire duration of anagen during which greying started (i.e. 2–6 years in human terminal scalp HFs (Dawber, 1997; Oh et al., 2016) and likely also that of the subsequent anagen phase. Therefore, prophylactic anti-greying interventions would be best initiated in the third or fourth decade of life, not after greying becomes easily visible and thus very extensive. These interventions must primarily (i) preserve and restore the pigmente function of a HFPU in a greying HF, and (ii) slow or delay and ideally block MSC damage for as long as possible to widen the therapeutic window.

Ideally, the latter strategy will include the development of topically applicable pharmacological MSC protectants that bolster antioxidant and DNA damage repair mechanisms and stimulate MSC proliferation. This would be particularly useful in patients with chemo- or radiotherapy-induced poliosis (Gao et al., 2019), since MSCs are vulnerable to genotoxic stress (Inomata et al., 2009; Gao et al., 2019). However, undesired MSC differentiation, such as by overexpressing endothelin-1 in the murine HF bulge (Takeo et al., 2016) or prolonged stimulation with thyroid hormones in human eHFSCs (Tiede et al., 2010), needs to be avoided. With regard to maintaining melanogenesis within the HFPU itself, topically applied serotonin-reuptake inhibitors like fluoxetine (Chéret et al., 2020), which exploit the existing capacity of epidermal and HF melanocytes to synthesize and metabolize serotonin (Slominski et al., 2002a; Slominski, Wortsman & Tobin, 2005), may be productive. Additionally, L-thyroxine (Van Beek et al., 2008), α-MSH or afamelanotide/melanotan (Paus et al., 2014), TRH (Gaspár et al., 2011) or theophylline, which increase intracutaneous melatonin synthesis (Bertolini et al., 2020), melatonin itself (Iyengar, 2000; Kobayashi et al., 2005; Fischer et al., 2008), and agents that stabilize ATM expression (Sikkink et al., in press) all deserve preclinical testing in organ-cultured greying human scalp skin.

VIII. CONCLUSIONS

(1) The biology of human HF greying represents a fascinating, interdisciplinary and translationally relevant biological research frontier at the intersection of pigmentation, gerontology, chronobiology and stem cell biology.

(2) The onset and perpetuation of defective/insufficient melanin production in the HFPU provides a superb model system for interrogating the molecular mechanisms of oxidative damage and tissue responses to oxidative stress in a complex human mini-organ.

(3) Human hair greying may represent a unique system – with an unusually visual phenotype – that permits examination of the psychobiological processes linking perceived stress and neuroendocrine and inflammatory signalling with the senescence of human melanocytes and their progenitor cells within their specialized tissue niches.

(4) All currently available evidence suggests that human hair greying is an anagen-dependent phenomenon that begins in the HFPU, most likely through insufficiently checked surges of intrafollicular ROS production resulting in progressive oxidative damage that disrupts tyrosinase activity, melanogenesis, melanosome transfer and HFPU melanocyte survival.

(5) In parallel with declining ROS scavenging and oxidative damage repair systems in the anagen hair matrix and declining local production of melanogenesis-promoting signals by keratinocytes of the HFPU, this progressively compromises the HF’s capacity to synthesize melanin and to transfer it to hair shaft keratinocytes.

(6) Given the ongoing ‘war on ageing’ (Sinclair & LaPlante, 2019) some of the ‘senolytics’ that are being explored in this context may also turn out to be useful in halting and reversing greying by enhancing and restoring the HFPU’s capacity to manage oxidative stress.
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