Published in final edited form as:

Pigment Cell Melanoma Res. 2015 March ; 28(2): 223–228. doi:10.1111/pcmr.12344.

Ectopic differentiation of melanocyte stem cells is influenced by genetic background

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Summary

Hair graying in mouse is attributed to the loss of melanocyte stem cell function and the progressive depletion of the follicular melanocyte population. Single-gene, hair graying mouse models have pointed to a number of critical pathways involved in melanocyte stem cell biology; however, the broad range of phenotypic variation observed in human hair graying suggests that additional genetic variants involved in this process may yet be discovered. Using a sensitized approach, we ask here whether natural genetic variation influences a predominant cellular mechanism of hair graying in mouse, melanocyte stem cell differentiation. We developed an innovative method to quantify melanocyte stem cell differentiation by measuring ectopically pigmented melanocyte stem cells in response to the melanocyte-specific transgene Tg(Dct-Sox10). We make the novel observation that the production of ectopically pigmented melanocyte stem cells varies considerably across strains. The success of sensitizing for melanocyte stem cell differentiation by Tg(Dct-Sox10) sets the stage for future investigations into the genetic basis of strain-specific contributions to melanocyte stem cell biology.

Keywords

melanocyte stem cell; genetic variation; hair graying; mouse; strain

Speculation that hair graying has a genetic etiology is found in literature dating back to nearly a century ago. In 1933, Rev. C. Ashlin West writes in a journal on human genetics, ‘Sir, For quite a number of years I have interested myself in the phenomena of “premature” grey hair, and am convinced that in many instances this kind of hair, the despair of the ladies, and the horror of business men, is not the outcome of any other than natural factors and that it is not a sign of prematurity, but rather the sign that germinal factors have been at work (West, 1933)’. Indeed, in humans, epidemiological data repeatedly demonstrate a variable genetic component to hair graying exemplified by differential onset with age based on race, with people of Asian and African descent graying later than Caucasians (Boas and Michelson, 1932; Keogh and Walsh, 1965; Panhard et al., 2012). Strong correlation between the amount of gray hair observed in monozygotic twins further suggests the influence of genetic factors on this phenotype (Gunn et al., 2009). Succumbing to age-related hair

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials and methods.

graying seems nearly inevitable, with greater than ninety percent of the world population exhibiting this phenotype by the age of sixty (Panhard et al., 2012). Yet despite this pervasiveness, the genetic variations responsible for differences in human hair graying remain unidentified.

Some insight into the genes and molecular mechanisms that influence hair graying has been gained using model organisms. Hair graying, or canities, is generally defined as a progressive replacement of pigmented hair with non-pigmented (gray) hair over time due to depletion of the follicular melanocyte population (Commo et al., 2004; Nishimura, 2005). In mouse, the prevalent mechanism by which this occurs is through the loss of melanocyte stem cell (McSC) maintenance. Predominantly, this is due to aberrant McSC differentiation, although hair graying in mouse is also attributed to failure of McSC survival or defects in the ability of McSCs to reenter the cell cycle (reviewed in Nishimura, 2011). Genes and pathways implicated in hair graying in mouse include melanocyte transcription factors (*Mitf*, *Sox10*), anti-apoptotic factors (*Bcl2*), paracrine signaling pathways (*Notch*, *Tgfb*, *Wnt/β-catenin*), signal transduction kinases (*B-raf*, *C-Raf*), hair follicle stem cell-associated collagens (*Col17a1*), and the DNA damage response (with ionizing radiation or mutations in *Atm*, *Xpd*; Aubin-Houzelstein et al., 2008; Harris et al., 2013; Moriyama et al., 2006; Nishimura, 2005; Nishimura et al., 2010a; Rabbani et al., 2011; Schouwey et al., 2007; Tanimura et al., 2011; Valluet et al., 2012). Yet beyond these single-gene models of hair graying, if we consider the complex and quantitative genetics of hair and skin pigmentation (Barsh, 1996; Pavan et al., 1995; Sturm, 2009), we should anticipate the participation of additional genetic variants in creating the phenotypic diversity of hair graying. Modifier genes in particular, which exhibit little or no phenotype in the absence of an independent conditioning mutation (Hamilton and Yu, 2012), are suggested to explain variation in human disease phenotypes and may play a similar role in hair graying in mouse.

We investigated whether genetic variation is sufficient to modify hair graying phenotypes in mouse by taking advantage of the hair graying mouse model Tg(Dct-*Sox10*) (Harris et al., 2013). Using the melanocyte-specific *Dct* promoter, Tg(Dct-*Sox10*) is expressed conditionally in melanocytes, and hemizygosity for this transgene (Tg(Dct-*Sox10*)/0) causes a twofold overexpression of the transcription factor *Sox10*. This causes McSCs to prematurely differentiate. Premature differentiation of McSCs in Tg(Dct-*Sox10*) animals is characterized by their production of ectopic pigmentation within the stem cell compartment of the hair upon their activation at anagen. Ectopically pigmented McSCs (EPMs) do not persist with hair cycling; once this stem cell pool is exhausted, subsequent hairs are devoid of pigment and are visualized as gray hairs. Further, hair graying susceptibility within the Tg(Dct-*Sox10*) line can be predicted by the frequency of hairs containing EPMs during the anagen hair growth stage (Harris et al., 2013). The presence of ectopic pigment within the McSC population is not limited to the Tg(Dct-*Sox10*) line, and EPMs are detected in other genetic mouse models at varying stages of hair growth. Interestingly, the production of EPMs at telogen does not adversely affect the McSC population (Chang et al., 2013), while the presence of EPMs at anagen is commonly associated with hair graying due to inadequate McSC maintenance (Aubin-Houzelstein et al., 2008; Harris et al., 2013; Inomata et al., 2009; Nishimura, 2005; Nishimura et al., 2010b; Rabbani et al., 2011; Tanimura et al.,

2011). An increase of EPMs within anagen-stage hairs is also seen with senile hair graying in mouse (Arck et al., 2006; Nishimura, 2005). These findings suggest that aberrant McSC differentiation observed specifically during active hair growth is a relevant hair graying-associated phenotype.

Hair graying in Tg(Dct-Sox10) animals is exacerbated by haploinsufficiency for the transcription factor *Mitf* (*Mitf^{ga9/+}*). Consequently, Tg(Dct-Sox10)/0; *Mitf^{ga9/+}* animals exhibit extensive ectopic pigment in their stem cell compartment and increased hair graying in comparison with Tg(Dct-Sox10)/0 animals, two phenotypes that are not observed in *Mitf^{ga9/+}* animals (Harris et al., 2013). These observations indicate that the severity of hair graying in this model is directly associated with an increased production of EPMs, and further suggests that EPM frequency in response to Tg(Dct-Sox10) is a genetically variable trait. In Tg(Dct-Sox10)/0 hemizygous animals, visible EPMs precede consequent hair graying by several weeks. Thus, EPM frequency within the Tg (Dct-Sox10) line provides a relatively early and quantifiable adult phenotype that can be used to assess whether natural genetic variation influences McSC biology in mouse.

To determine the contribution of genetic background to EPM frequency, we established a sensitized screen. Inbred females are mated to Tg(Dct-Sox10)/0 males to produce progeny that are sensitized, or predisposed, to hair graying by the presence of the Tg(Dct-Sox10) transgene. This type of sensitized screen allows us to detect modifier effects, or mutations, contributed by the inbred background that produce small changes in McSC differentiation and that would otherwise be undetectable in wild-type animals. Wild-type and hemizygous Tg(Dct-Sox10) F1 animals were generated by mating hemizygous C57BL/6J-Tg(Dct-Sox10) males to females from six inbred lines: C57BL/6J, C3H/HeJ, DBA/1J, BALB/cJ, 129S6/SvEvTac, and FVB/NTac (see Table 1 for mating scheme and F1 progeny abbreviations). These inbred lines were selected based on their representation of genetically divergent groups (Frazer et al., 2007; Petkov et al., 2004), and their inclusion in a study of hair graying sensitivity in response to ionizing radiation (C57BL/6 and DBA/1J, Potten, 1969). We developed a simple method to assess hairs containing EPMs during hair growth using histology and light microscopy (see Data S1). F1 animals are plucked at approximately nine weeks of age within telogen of the first adult hair cycle (Müller-Röver et al., 2001) and assessed for EPMs 7 days later during midanagen. Cryosections of anagen-stage skin are viewed under bright field microscopy, and the numbers of hairs containing EPMs are quantified. Hairs are evaluated for the presence of EPMs using the following criteria: (i) The stem cell compartment, or lower permanent portion of the hair, must be visible and extend from the opening of the sebaceous gland duct to the junction between the dermis and subcutis (indicated in Figure 1A–F; as defined in Aubin-Houzelstein et al., 2008) and (ii) hairs are deemed positive for EPMs if any pigment is visible in this region, independent of the amount of pigmentation. Additionally, all hair types are evaluated (zigzag, guard, awl, and auchene), and both males and females are included in the analysis.

General inspection of hairs from F1 hybrids indicates that Tg(Dct-Sox10) induces the production of EPMs in all genetic backgrounds but to a varying degree (Figure 1). The extent of ectopic pigment exhibited by individual hair follicles also varies widely, and no particular pattern of EPMs is observed among hairs from the same strain or between strains

(Figure 1A'–F'). All animals produce hairs that are pigmented and in a similar hair growth stage, ranging from anagen-IIIc to anagen-V (Figure 1A–F). This suggests that any differences observed in EPM frequency between strains are likely not due to a complete lack of McSCs or a failure in McSC activation, as pigmented hairs indicate both the presence of McSCs and McSCs that are competent to produce functional progenitors. This also indicates that variances in EPM phenotypes are not the result of overt differences in hair growth rates. The color of EPMs in Tg(Dct-Sox10)/0 F1 hybrids appears independent of coat color. All EPMs display similar brown/black, eumelanin-like pigment as seen in C57BL/6J-Tg(Dct-Sox10)/0 animals regardless of whether the animal carries a mutation in the *nonagouti* gene (C3B6F1, 129B6F1, FVBB6F1), *Tyrp1* gene (D1B6F1, FVBB6F1), or *Tyr* gene (CB6F1, FVBB6F1) (Figure 1, A'–F').

In support of our hypothesis that natural genetic variation contributes to differences in McSC differentiation, the percentage of hairs containing EPMs in Tg(Dct-Sox10)/0 animals varies strikingly based on genetic background (Figure 1G). As predicted from our previous study (Harris et al., 2013), we confirm in each strain that the occurrence of EPMs is significantly associated with the presence of the Tg(Dct-Sox10) transgene (Fisher's exact test, $P < 0.0001$; Table 2). However, comparing this association between strains indicates that the strain-specific response to the transgene is not homogenous (Woolf's method, $P = 0.0002$). When considering the odds ratios of individual strains, the effect can be ordered from largest to smallest: B6, CB6F1, FVBB6F1, C3B6F1, 129B6F1, and D1B6F1 (Table 2). The B6 strain in particular exhibits a 2.2- to 4.13-fold higher association between EPMs and Tg(Dct-Sox10) in comparison with all other strains, excluding CB6F1. These observations confirm that strain-specific natural genetic variation influences McSC differentiation when sensitized for hair graying by Tg(Dct-Sox10) hemizygosity.

This study supports the idea that a specific cellular mechanism involved in hair graying, McSC differentiation, is a genetically modifiable trait that encompasses a range of phenotypic severity. While it is unknown how much McSC differentiation contributes to overall variability in hair graying, the predominance of this trait in hair graying mouse models suggests that further investigation into the modifier genes and pathways that regulate this process is warranted. Although we anticipate potential genetic variants identified by this screen to be involved in driving or preventing McSC differentiation, changes in the frequency of EPMs may also reflect the influence of genetic background on other cellular mechanisms equally interesting and relevant to the McSC, such as initial establishment of McSCs, overall McSC number, McSC proliferation or survival, and migration of McSC progenitors.

Inbred mouse lines represent a limited level of genetic heterogeneity in comparison with the diversity observed in humans; however, we demonstrate here that inbred lines provide sufficient genetic variation to affect this particular phenotype. There are no similar studies that evaluate the variability of hair graying-associated cellular phenotypes in other animals, although additional examples exist to support the possibility that genetic variation influences hair graying in general. For instance, age-related graying in horses is attributed to an intronic duplication in the *syntaxin-17* gene, yet Connemara horses gray much later than horses of other breeds carrying the same mutation. This suggests that the presence of modifier genes

in the Connemara breed provides resistance to this phenotype (Rosengren Pielberg et al., 2008; Sundström et al., 2012). As a second example, ionizing radiation (IR) causes hair graying in mice, and susceptibility to IR varies by genetic background; strong F mice show increased depletion of follicular melanocytes after IR in comparison with DBA/1 and C57BL/6 mice (Potten, 1969).

Whether the known genes and pathways that cause hair graying in mouse or horse participate in age-related hair graying in humans is still unknown. However, one feature of hair graying that is shared between mouse and human is an increase in oxidative stress and DNA damage (reviewed in Tobin, 2011). Gray hairs in humans exhibit millimolar quantities of H₂O₂, reduced catalase levels, and the presence of oxidatively damaged DNA bases (Arck et al., 2006; Kausar et al., 2010; Wood et al., 2009). In mice, initiating similar genotoxic stress by H₂O₂ or IR activates the DNA damage response, which in turn drives McSC differentiation and hair graying (Inomata et al., 2009; Ueno et al., 2014). Differentiation of McSCs is a phenotype that is observed in both aged human and mouse hairs and suggests that accumulating DNA damage within McSCs over time is a common mechanism for age-related hair graying (Arck et al., 2006; Nishimura, 2005). Thus, as a complement to the approach presented here, investigating the modifier genes that influence the response to oxidative stress in McSCs in mouse may identify additional pathways for stem cell maintenance that are relevant across species.

Modifier genes have garnered increasing interest because of their potential role in the genetic plasticity of human disease, and because the advent of new genomic tools has made their molecular validation feasible (Hamilton and Yu, 2012). In particular, mouse genome resequencing efforts have paved the way to investigate the contribution of naturally derived mutations to strain-specific effects on phenotype (Frazer et al., 2007; Keane et al., 2011). Our report of the interstrain variability in McSC differentiation when sensitized for hair graying by genetic manipulation of *Sox10* provides the first step toward identifying novel genetic variants involved in McSC biology. We confirm that genetic background indeed influences the frequency of hairs that exhibit ectopically pigmented McSCs and identify a number of strains that appear resistant to McSC differentiation. Additionally, based on the critical role of SOX10 in supporting the follicular melanocyte lineage and its role in melanocyte development and function (Harris et al., 2010, 2013), the use of this particular transgene may also allow us to gain insight into upstream regulators of *Sox10* expression. Future identification of the modifier genes involved in this process will further elucidate how the McSC population is regulated and may broaden our understanding of the molecular mechanisms involved in hair graying.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

For advice, we thank the Pavan laboratory members and give special mention to L. Baxter for editing the manuscript. For technical and mouse husbandry assistance, we thank A. Incao. This research was funded by the

Intramural Research program of NIH/NHGRI (to DL, DW, WP) and by a NIH K99/R00 Pathway to Independence Award (to MH).

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Significance

Few reports exist comparing the frequency of cellular phenotypes associated with hair graying in mice of different strains. Perhaps this is a consequence of anecdotal observations that hair graying occurs only in C57BL/6 mice, a conclusion likely attributable to the primary use of young mice in laboratory research, and the fact that hair graying is difficult to appreciate on a non-black coat color. As hair graying is studied largely in C57BL/6 mice, the influence of natural variation on cellular mechanisms that produce this phenotype is not appreciated. Here, we provide the first evidence of strain-specific effects on melanocyte stem cell differentiation in mouse.

Figure 1.

Tg(Dct-Sox10) induction of EPMS is independent of genetic background and induces a variable frequency of hairs with EPMS in F1 progeny. (A–F) 10× brightfield images of skin from female Tg(Dct-Sox10)/0 F1 progeny described in Table 1. The dotted line outlines an example of one hair, and error bars mark the region between the sebaceous gland (upper end) and dermal–subcutis border (lower end) where McSCs reside within each hair. The white boxes indicate the region of one hair containing EPMS and are enlarged in (A'–F'). Scale bar = 100 μm. (A'–F') 40× brightfield images showing magnified view of EPMS. Bracket indicates region of hair occupied by EPMS. Scale bar = 20 μm. (G) EPM severity in F1 animals is represented as the percentage of hairs exhibiting EPMS out of the total hairs analyzed. Individual data points represent one animal, and vertical bars indicate the mean for that genotype. Triangles and circles designate Tg(Dct-Sox10)/0 (Tg/0) and wild-type (+/+) animals, respectively.

Table 1

Mating scheme for F1 cross

Cross (female × male)	F1 abbreviation	N ^a (Tg/0, +/-)
C57BL/6J × C57BL/6J-Tg(Dct-Sox10)/0	B6	14, 10
C3H/HeJ × C57BL/6J-Tg(Dct-Sox10)/0	C3B6F1	8, 7
129S6/SvEvTac × C57BL/6J-Tg(Dct-Sox10)/0	129B6F1	7, 7
FVB/NTac × C57BL/6J-Tg(Dct-Sox10)/0	FVBB6F1	10, 10
DBA/1J × C57BL/6J-Tg(Dct-Sox10)/0	D1B6F1	8, 8
BALB/CJ × C57BL/6J-Tg(Dct-Sox10)/0	CB6F1	9, 9

^aN refers to the number of F1 progeny analyzed from the preceding cross.

Table 2
Proportion of hairs with EPMs in Tg(Dct-Sox10) and wild-type animals stratified by strain

Strain	Genotype ^a	Hairs ^b		Total	Odds ratio	95% CI	Fi exact
		With EPMs	W/out EPMs				
B6	Tg/0	775	341	1116	42.26	28.11, 63.53	P < 0.00001
	+/+	27	502	529			
C3B6F1	Tg/0	382	324	706	14.46	10.34, 20.21	P < 0.00001
	+/+	46	564	610			
129B6F1	Tg/0	246	212	458	14.08	9.870, 20.08	P < 0.00001
	+/+	45	546	591			
FVBB6F1	Tg/0	223	481	704	18.95	11.26, 31.90	P < 0.00001
	+/+	16	654	670			
D1B6F1	Tg/0	77	487	564	10.23	5.238, 19.98	P < 0.00001
	+/+	10	647	657			
CB6F1	Tg/0	133	555	688	30.83	13.51, 70.39	P < 0.00001
	+/+	6	772	778			
				Woolf Heterogeneity Test		P = 0.0002	

^a Tg/0 indicates animals hemizygous for the Tg(Dct-Sox10) transgene.
^b Counts represent summed totals of each category across the biological replicates (N, Table 1) for the strain indicated.