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Coupling of the radiosensitivity of melanocyte stem cells to their dormancy during the hair cycle

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Summary

Current studies have revealed that stem cells are more radiosensitive than mature cells. As somatic stem cells are mostly kept in a quiescent state, this conflicts with Bergonie and Tribondeau’s law that actively mitotic cells are the most radiosensitive. In this study, we focused on hair graying to understand the stress-resistance of melanocyte stem cells (McSCs). We used Dct-H2B-GFP transgenic mice which enables the stable visualization of McSCs and an anti-Kit monoclonal antibody which selectively eradicates amplifying McSCs. The results demonstrate that quiescent McSCs are rather radiosensitive, but the coexistence of non-quiescent McSCs provides the stem cell pool with radioresistance. The irradiated quiescent McSCs prematurely differentiate in the niche upon their activation without sufficiently renewing themselves for cyclic hair pigmentation. These data indicate that tissue radiosensitivity is largely dependent on the state of somatic stem cells under their local microenvironment.

Introduction

Stem cells reside in most adult mammalian tissues where they maintain normal tissue homeostasis and participate in tissue repair and regeneration in response to different types of stresses and damages (Li and Clevers, 2010; Weissman, 2000). Tissues, such as bone marrow, intestine, and epidermis, show functional declines and diminished regenerating capabilities with aging, and these regenerative tissues are known to be sensitive to ionizing radiation (IR; Stone et al., 2003). In 1906, Bergonie and Tribondeau proposed a law describing the radiation sensitivity for all cells in the body. Their law maintains that actively mitotic undifferentiated cells are the most susceptible to damage from IR (Bergonie and Tribondeau, 1959). Consistently, a number of studies with different stem cell systems have demonstrated that immature stem cells are more radiosensitive than mature cells (Barker et al., 2007; van der Meer et al., 1992; Potten et al., 1997). Thus, it has been believed for a long time that the radiosensitivity of actively mitotic stem cells underlies the radiosensitivity of regenerative tissues (Bergonie and Tribondeau, 1959). However, current stem cell studies have revealed that somatic stem cells are

Significance

Current studies have revealed that stem cells are more radiosensitive than mature cells. However, somatic stem cells are mostly kept in a quiescent state, which conflicts with the conventional dogma that actively mitotic cells are radiosensitive. Here, we focused on hair graying to understand the stress-resistance of melanocyte stem cells (McSCs). We selectively eradicated amplifying McSCs in Dct-H2B-GFP transgenic mice which enabled the stable visualization of McSCs and successfully demonstrate that quiescent McSCs are radiosensitive. The coexistence of non-quiescent McSCs in the niche ensures the resistance of the McSC pool to different kinds of stresses to prevent hair graying.
generally slow-cycling cells and are mostly kept in a quiescent state (Buczacki et al., 2013; Fuchs, 2009; Li and Clevers, 2010; Orford and Scadden, 2008). Furthermore, our group and others recently found that somatic stem cells under genomic stress commit to differentiation without renewing themselves (Inomata et al., 2009; Wang et al., 2012). Those findings raised new questions of whether all immature stem cells are radiosensitive or not, whether they lose their immaturity or die by apoptosis, and whether actively cycling stem cells are really radiosensitive or not. The biological significance for the regulation of the quiescent state versus the cycling state of somatic stem cells and its correlation with the stress-resistance of stem cells and tissues has not yet been clarified.

The hair follicle is a skin appendage organ which repeats its cyclic regeneration. Stem cells in this mini-organ are periodically activated in every hair cycle (Fuchs, 2007). Melanocyte stem cells (McSCs), which serve as a reservoir for melanocytes (pigment cells) required for hair pigmentation (Nishimura et al., 2002), are cyclically activated during each hair cycle. They reside in the bulge (Bgl–sub-bulge (sBg) area of mammalian hair follicles and directly contact hair follicle stem cells (HFSCs) which serve as the functional niche for McSCs needed to continue to grow pigmented hair (Nishimura, 2011; Nishimura et al., 2002; Tanimura et al., 2011). The behavior of McSCs is tightly synchronized with progression of the hair cycle under physiological conditions, but McSC maintenance becomes incomplete with aging or by genomic stress (Inomata et al., 2009; Nishimura et al., 2010). In response to excessive but non-lethal levels of genomic stress, such as 5 Gy IR, McSCs differentiate into pigment-producing melanocytes ectopically in the niche without renewing themselves (Inomata et al., 2009). Somatic stem cells are generally maintained in tissues for a long time by resisting different genotoxic stresses such as those caused daily by reactive metabolic by-products as well as by occasional stresses, such as IR and chemotherapeutic drugs. However, how stem cell pools are sustained in tissues by resisting those different stresses or whether all stem cells are homogeneous with the same stress-resisting capabilities has not yet been studied in any stem cell system.

In this study, we took advantage of the Dct-H2B-GFP system, a new tagging system for the melanocyte lineage which we devised to stably visualize McSCs even when they are in a deep quiescent state. We succeeded in chasing the fate of McSCs throughout the hair cycle and found that McSCs show different radiosensitivities depending on their state during each hair cycle.

Results

Cyclic radioresistance of McSCs during the hair cycle

Melanocyte stem cells are essential for cyclic hair pigmentation. The incomplete maintenance of McSCs upon aging and due to genomic stress results in hair graying which progresses every hair cycle (Inomata et al., 2009; Nishimura et al., 2005). Hair follicles repeat cyclic hair regeneration by altering the phases of growth (anagen), regression (catagen), and rest (telogen) during each hair cycle. Mice irradiated with a dose of 5 Gy IR show homogeneous hair graying in most coat areas as we previously described (Inomata et al., 2009), but occasionally some other areas still maintain the original coat color without showing significant hair graying (Figure 1A, arrow). As most pelage hair follicles are resting at the telogen phase, which is the longest phase during the hair cycle in adult mice, but those in other areas are not, we hypothesized that the radiosensitivity of hair follicles varies at different hair cycle stages. To test this possibility, we irradiated C57BL/6 mice who had their hair follicles synchronized by the hair depilation method at different hair cycle stages after hair cycle induction at 7 weeks of age when all hair follicles are arrested at telogen (Muller-Rover et al., 2001; Figure 1B). As shown in Figure 1C, hair was significantly grayed when catagen–telogen hair follicles were irradiated. In sharp contrast, newly grown hair did not show any significant hair graying when anagen hair follicles were irradiated. These data indicate that hair follicles show different sensitivities to IR at different hair cycle stages. Similar radiosensitivities were observed during spontaneous hair cycle progression as well. We found that hair grayed significantly when C57BL/6 mice were irradiated at 19–22 days after birth when hair follicles on the dorsal skin are mostly synchronized at catagen–telogen phase but not at anagen phase (Figure S1). It is notable that the graying of hair follicles was observed conspicuously after the second hair cycle (Figure 1C). As most melanocytes in the hair follicle bulb are newly supplied from McSCs in the Bg–sBg area during early anagen at the beginning of each new hair cycle, these data suggest that McSCs in the Bg–sBg area show different radiosensitivities at different hair cycle stages.

We previously found that damaged McSCs in telogen hair follicles are depleted after 5 Gy IR (Inomata et al., 2009). It is thus possible that McSCs are radioresistant at anagen phase but not at other stages of the hair cycle. In previous studies, fate analysis of McSCs has been limited to anagen phase because of the lack of a perfectly stable cell marking system. Kit and Dct are useful markers of melanocytes including McSCs and Kit and Dct are expressed in melanoblasts/melanocytes of anagen hair follicles (Botchkareva et al., 2001. However, their expression level is often diminished after late anagen (AnagenVI) until hair follicles enter the anagen phase (Figure 2B and data not shown). Dct encodes the enzyme dopachrome tautomerase (also known as TRP-2), a marker for the melanocyte lineage, and we have used Dct-lacZ transgenic (tg) mice (Mackenzie et al., 1997) to visualize melanoblasts and melanocytes (Inomata et al., 2009; Nishimura et al., 2002). LacZ signals also become dimin-
ished from Anagen VI (Figure 2B) and are maintained at a low/undetectable level during the catagen/telogen phase, which thus does not allow the accurate quantification of McSCs at different hair cycle stages. To solve this problem, we generated mice which stably express a Histone–GFP fusion protein (H2B–GFP) in melanocyte lineage cells under control of the Dct-promoter (Figure 2A). In those mice, GFP+ cells were stably found within the Bg–sBg area throughout the hair cycle and they co-express Kit and Dct-lacZ except during the long telogen phase (Figure 2B, C). Thus, Dct-H2B-GFP tg mice enable the quantification of McSCs, which we previously defined as immature unpigmented melanoblasts located within the Bg–sBg area, at all hair cycle stages (Nishimura et al., 2002). Although some GFP+ cells were found in the dermis outside of the basement membrane of the Bg–sBg area as well, most of those cells co-express neuronal cell markers such as TuJ1. This enables us to clearly distinguish McSCs inside the Bg–sBg area from neuronal cells located outside of the basement membrane (Figure 2C). As Mitf<sup>−/−</sup> mutant mice, which completely lack melanocyte lineage cells (Zimring et al., 1996), lost GFP+ cells in the Bg–sBg area but retain dermal GFP+TuJ1+ cells (Figure S2A), we assumed that these neuronal cells, which express H2B–GFP due to transient activation of the Dct-promoter during development, can be clearly distinguished from McSCs located within the Bg–sBg area. These findings allowed us to establish a system which is useful for the accurate and stable visualization and quantification of McSCs. Importantly, we found that the number of GFP+ cells in the Bg–sBg area is normally maintained almost at the same level throughout the hair cycle (Figure S2B). As this cellular tagging with the H2B–GFP fusion protein is quite stable even in melanoblasts/melanocytes in a deep quiescent state, we took advantage of this McSC monitoring system to trace the fate of

Figure 1. Cyclic radioresistance to hair graying during the hair cycle. (A) Coat colors of 6-month-old C57BL/6 wild-type mice with or without 5 Gy IR. Hair graying was induced almost homogeneously on the back except for some intact areas (arrow) when irradiated at 7 weeks after birth. (B) Schematic for the distribution of melanocyte lineage cells at different hair cycle stages. The hair cycle was induced synchronously by the depilation of telogen follicles at 7 weeks after birth. D0-1: resting phase (Telogen); D2–D17 growing phase (Anagen); D18–24: regressing phase (Catagen); D25–: resting phase (Telogen) (C) The coat color of C57BL/6 wild-type mice after 5 Gy IR at different hair cycle stages induced by depilation. Hair depilation was performed every 30 days when hair follicles are synchronized at the telogen phase. Photos were taken at the end of each hair cycle.
McSCs after various kinds of stress. First, we irradiated mouse skin at different hair cycle stages with 5 Gy IR to quantify McSCs in the niche using Dct-H2B-GFP mice (Figure 2D). We found that GFP+ cells in the Bg–sBg area are decreased in number when irradiated at telogen phases but are well maintained when irradiated at most of the anagen phase (Figure 2E, F and S2C). These data indicate that McSCs in anagen hair follicles are resistant to IR.

Spatiotemporal regulation of McSC status during the hair cycle

Next, to explore the correlation between the radioresistance of McSC pools and the McSC activation status, we carefully examined the expression of cell cycle markers, including Ki67, Mcm2, and BrdU incorporation, in Dct-H2B-GFP/LacZ-expressing McSCs. Although our previous study showed that McSCs in the Bg–sBg area can be labeled by BrdU only at early anagen phase (Nishimura et al., 2002), the exact stage when McSCs are proliferating has not yet been determined. At the telogen stage, almost all hair follicles are Ki67/Mcm2-negative (Figure 3A, B). At day 2 after depilation (D2), hair follicles are synchronized at anagenI and Ki67/Mcm2-positive cells were found only in the sBg area (hair germ) but not in the Bg area of those hair follicles (Figure 3C, D). At D3–D4 (AnagenII~III), many more Ki67/Mcm2-positive melanoblasts were detected not only in the sBg area (hair germ) but also in the Bg area (Figure 3E–H). At D6 (anagenVI), Ki67/Mcm2 expression was found in the Bg–sBg area with less frequency in the Bg area (Figure 3I, J). Mcm2 expression by GFP+ cells in the Bg and sBg areas diminished gradually at the end of anagen (Figure 3K, L) and then became undetectable at D10 or later. At D20–D30 (catagen-next telogen), the entire hair follicle became Ki67/Mcm2-negative (data not shown). Notably, as Mcm2 positivity was found in approximately half [51.6% (SD = 6.7)] of the GFP+ cells in the Bg–sBg area during...
These observations indicate that McSCs in the Bg area but also in the sBg area when labeled at D3. On the other hand, BrdU uptake was found not only in the sBg area when labeled at D2 (Figure 4Ab). These remaining cells were almost all Mcm2+/C0 cells; arrowheads, KI67− or Mcm2− cells). Bg, bulge; sBg, subbulge. Scale bars represent 50 μm.

Figure 3. The distribution of cycling cells in hair follicles during anagen progression. Immunohistochemical analysis of the cell cycle markers KI67 (A, C, E, G, I, K) and Mcm2 (B, D, F, H, J, L) in Dct-LacZ tg mice and in Dct-H2B-GFPtg mice, respectively, at different stages during anagen progression (arrows, KI67+ or Mcm2+ cells; arrowheads, KI67− or Mcm2− cells). Bg, bulge; sBg, subbulge. Scale bars represent 50 μm.

eyearly-mid-anagen phase (D3 and D4), this suggests that approximately half of them are kept in a quiescent state and the other half of them are activated. To further examine the distribution of McSCs at S-phase, we performed BrdU-labeling experiments. McSCs in the Bg–sBg areas were labeled from D2 to D5 after hair cycle induction and the distribution of BrdU-labeled cells were similar to that of Ki67/Mcm2-positive cells (Figure 4A). The activation status of McSCs and their IR sensitivity during the hair cycle is summarized in Figure 4C.

It was notable that BrdU−/LacZ+ cells were found only in the sBg area when labeled at D2 (Figure 4Ab). On the other hand, BrdU uptake was found not only in the sBg area but also in the Bg area when labeled at D3 (Figure 4Ac). These observations indicate that McSCs in the sBg area are activated earlier than McSCs in the Bg area, suggesting that the two populations are activated differentially at distinct stages in distinct areas. To examine the difference in the fate of the populations in the Bg and sBg area, we performed BrdU pulse–chase experiments after a single BrdU pulse at D2 or D3. At D2, BrdU-positive cells were observed only in the hair germ (the sBg area), but subsequently BrdU-positive cells were found mostly in the hair matrix of the down-growing hair bulb with progressively diminished BrdU retention (Figure 4Ba–c,g). Although some BrdU-positive cells were retained over hair cycles in the sBg area, the number was comparably limited (Figure 4Bi,k). This indicated that many sBg melanoblasts, which are proliferating in the hair germ (sBg) at D2, directly provide their progeny to the hair matrix. On the other hand, when labeled at D3, many BrdU-positive cells were found both in the sBg area and in the Bg area just after BrdU administration. The label was retained abundantly in both the Bg and sBg areas without reduction in number (Figure 4Bd–f,h). In the following hair cycle (2nd–D5), BrdU-retaining cells were similarly observed in the niche when labeled at D3 (Figure 4Bj,k). These data suggest that McSCs proliferating mainly in the Bg area at D3 efficiently renew themselves to maintain the stem cell pool for a longer time, probably as a ‘long-term stem cell pool’.

Non-quiescent McSCs can be distinguished from quiescent McSCs by their Kit dependency for cell survival

As many McSCs are at the non-quiescent cycling status during the IR-resistant anagen phase, we hypothesized that quiescent McSCs are radiosensitive. To test this, we took advantage of the anti-Kit neutralizing antibody ACK2, which is able to deplete cycling melanoblasts/melanocytes during melanocyte development (Nishikawa et al., 1991; Nishimura et al., 2002; Okura et al., 1995). When ACK2 was given at early anagen phase (D1, D3, and D5), which coincides with the phase of McSC activation and renewal (Figure 5A), all melanocytes in the hair matrix were eradicated (Figure S3Ab) and all newly grown hairs were unpigmented (Figure 5Ba). On the other hand, approximately half of GFP+ melanoblasts survived in the Bg–sBg area after ACK2 treatment (Figure 5C, S3Ab). These remaining cells were almost all Mcm2−, while Mcm2+/GFP+ cells have disappeared (Figure 5D). As some GFP+ cells contain cleaved Caspase-3 in the Bg–sBg areas just after the treatment with ACK2 (Figure S5E), these data indicate that proliferating McSCs were successfully deleted by the induction of apoptosis with ACK2 treatment. Notably, the number of McSCs in the Bg–sBg area was normalized to the control level in the following hair cycle (Figure 5C), suggesting that quiescent McSCs remaining in the niche compensate for the loss of the McSC pool during the following hair cycle (Figure S5D). In fact, mature melanocytes also normally reappeared in the hair bulb (Figure S3Ac) and the newly grown hair became...
fully repigmented in the following hair cycle (Figure 5Bb). Thus, McSCs in anagen hair follicles can be classified into two groups, a Kit-dependent non-quiescent McSC pool and a Kit-independent quiescent McSC pool which can survive independently of Kit.

**Kit-dependent McSCs in the non-quiescent state are radioresistant to maintain them in an undifferentiated state**

To examine the radioresistance of quiescent versus non-quiescent McSCs of anagen hair follicles, we took advantage of ACK2, which does not influence the hair cycle independently from active SCF–Kit signaling. As shown in Figure 6B, GFP+ cells in the niche did not decrease in number in the following hair cycles. This indicates that quiescent McSCs are significantly sensitive to maintain them in an undifferentiated state.

**Radioresistance of melanocyte stem cells**

![Radioresistance of melanocyte stem cells](image)

Figure 4. Spatiotemporal regulation of McSC status during the hair cycle. (A) The spatial distribution of replicating cells in hair follicles at different hair cycle stages. (a–e) BrdU incorporation by LacZ+ cells in the stem cell niche was analyzed in hair follicles at different hair cycle stages in Dct-lacZtg mice larrows, BrdU+ cells; arrowheads, BrdU+ cells. (f) Quantitative analysis of replicating McSC number in the niche during anagen progression. Shown is the number of BrdU+LacZ+ cells in the Bg-sBg area of hair follicles during telogen-anagen progression. (B) Differential distribution of replicating McSCs in the stem cell niche at early anagen phase. BrdU-incorporation into hair follicles was analyzed after a single BrdU administration into Dct-lacZtg mice on D2 (a–c) and D3 (d–f) after hair cycle induction. The skin was analyzed at 1 h, 4 h and 24 h after administration. (g, h) Quantitative chronological analysis of the number of replicating McSCs in Anagen I and II hair follicles, respectively. The number of BrdU+LacZ+ cells located in the Bg-sBg area of hair follicles was counted at different time points. (i, j, k) BrdU-retention in hair follicles at mid-anagen of the following hair cycle on D35 (2nd-D5 after hair cycle induction). BrdU administration into Dct-lacZtg mice on D2 (i) and D3 (j). (k) The number of label-retaining McSCs. BrdU+LacZ+ cells located in the Bg-sBg area of hair follicles was counted at mid-anagen of the following hair cycle. More label-retaining McSCs were found in hair follicles treated on D3. (C) Schematic for the distribution of cycling and quiescent McSCs in hair follicles at different hair cycle stages and the correlation of the McSC activation status with their IR-resistance. Bg, bulge; sBg, sub-bulge. Scale bars represent 50 μm.

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insufficient numbers of mature functional melanocytes for hair pigmentation (shown schematically in Figure 6D). These findings again demonstrate that quiescent McSCs are radiosensitive.

Next, we performed chronological fate analysis of the radiosensitive McSCs. We reported previously that a number of McSCs irradiated at telogen phase ectopically differentiate into mature melanocytes which contain abundant melanin pigment within the Bg–sBg area during the following mid-anagen after which those mature cells selectively disappear from the niche with retention of other immature McSCs in a few days when hair follicles start regressing during catagen phase in a synchronized manner (Inomata et al., 2009). In the following hair cycles, newly pigmented GFP+ cells appeared within the niche again at mid-anagen (2nd D5; Figure S4A, F). As only a part of the McSC pool in the niche is activated in every hair cycle, some of the remaining McSCs may ectopically differentiate in the niche at the time of their reactivation during anagen after which these cells may be specifically eliminated from the niche by unknown mechanisms. This explains why the ectopically differentiated melanocytes are found in the niche repeatedly at mid-anagen of every hair cycle.

To test whether the ectopic differentiation of McSCs is coupled with their cyclic activation during anagen, we examined the correlation between ectopic pigmentation of McSCs and their cell cycle state after irradiation at anagen phase using immunostaining for Mcm2 and detection of melanin pigment by DIC imaging. We sampled the skin of irradiated mice a few days after IR and at the following mid-anagen (Figure S4B–E). In hair follicles irradiated at late anagen (no ACK2, 5 Gy on D9), in which both non-quiescent and quiescent McSCs still exist, only a limited number of pigmented GFP+ cells were observed a few days after irradiation (Figure S4B, G), but a large number of pigmented GFP+ cells were observed in the following mid-anagen (Figure S4C, G). On the other hand, GFP+ cells in hair follicles irradiated after ACK2 treatment (ACK2 + 5 Gy D9) were all maintained in the unpigmented immature state in the niche just after IR (Figure S4D, H) but showed significant ectopic differentiation in the niche during the following mid-anagen phase (Figure S4E, H). As these ectopically pigmented GFP+ cells were all Mcm2-positive (Figure S4C, E), these findings indicate that McSCs ectopically differentiate within the niche only when they are reactivated for cell cycle entry (Figure S4I–K). Importantly, more unpigmented GFP+ cells (McSCs) were maintained in the Bg–sBg area of hair follicles treated only with IR in the following hair cycle (Figure S4C, arrowhead) compared with those in hair follicles treated with both ACK2 and IR. This was consistent with the result that mice irradiated after ACK2 treatment lost McSCs more significantly (Figure 6B) resulting in progressive hair graying in the following hair cycles. On the other hand, mice treated only with IR or ACK2 during anagen did not show significant changes in
coat color (Figure 6C) with significant recovery of stem cell number (Figure 6B). The results of the chronological analysis and histological observation are summarized in Figure S5. Our data demonstrate that quiescent McSCs, both in anagen and telogen hair follicles, are radiosensitive, but the coexistence of non-quiescent McSCs in the shared niche makes the stem cell pool radioresistant. Regulation of McSC states ensures the maintenance of McSC pools for cyclic hair pigmentation

In general, cells in tissues are exposed to various types of endogenous and exogenous genomic stress, including intrinsic reactive oxygen species, oxidative chemicals, and IR (Branzei and Foiani, 2008). In contrast to IR, which we found preferentially depletes quiescent McSCs, many chemotherapeutic drugs such as 5FU selectively damage amplifying cells. 5FU is an antimetabolite and its major cytotoxicity occurs during S-phase (Longley et al., 2003). We next analyzed the fate of McSCs treated with both IR and 5FU to examine the outcome of concomitant depletion of both quiescent and cycling McSC populations (Figure 7A). Mice were treated with IR and/or 5FU at D3 when many McSCs are at S-phase with maximal BrdU uptake. The number of McSCs in mice treated with IR...
and 5FU decreased significantly, but the number of McSCs of mice treated with IR or 5FU showed a mild decrease in the following hair cycles (Figure 7B). Consistently, mice treated with IR showed only slight hair graying and mice treated with 5FU did not show significant coat color changes, but mice treated with both IR and 5FU showed hair graying (Figure 7C). These data indicate that quiescent McSCs or non-quiescent McSCs can survive after selective depletion of either population by a certain type of genomic stress. Taken together, our data demonstrate that the coexistence of both quiescent and non-quiescent populations in the shared niche provides tissues with strong stress-resistance for maintenance of the stem cell pool and contributes to sustained tissue homeostasis (shown schematically in Figure 7D).

Discussion

The accumulation of somatic DNA damage is considered to be a main cause of aging and cancer in multicellular organisms (Inomata et al., 2009; Nijnik et al., 2007; Rossi et al., 2007; Ruzankina et al., 2007). The maintenance of stem cell pools in tissues has been considered to be enabled by maintaining the stem cells at the quiescent state which minimizes endogenous stress caused by cellular respiration and DNA replication (Orford and Scadden, 2008). X-ray irradiation is one of the major threats to genomic integrity and has been considered to target proliferating cells as Bergonie and Tribondeau proposed (Bergonie and Tribondeau, 1959). While the radiosensitivity of human and murine somatic stem cells, such as hematopoietic stem cells, intestinal stem cells, and spermatogonial stem cells, has been reported (Barker et al., 2007; van der Meer et al., 1992; Milyavsky et al., 2010; Mohrin et al., 2010; Potten et al., 1997), it has been unclear whether all somatic stem cells are equally sensitive to IR or have different sensitivities depending on their cell state. In this study, we used Dct-H2B-GFP transgenic mice to efficiently and stably visualize McSCs and treatment with ACK2 to selectively eradicate amplifying melanocytic cells to demonstrate that McSCs at the quiescent state are particularly sensitive to IR. The irradiated quiescent McSCs prematurely differentiate into pigment-producing melanocytes without undergoing self-renewal or apoptosis, but McSCs at the non-quiescent state are rather radioresistant. As the activation status and fates of McSCs are largely dependent on the niche microenvironment including hair follicle stem cells (Nishimura et al., 2002; Rabbani et al., 2011; Tanimura et al., 2011), the different radiosensitivity of McSCs at different cell cycle states might be governed by their local niche microenvironment, which is dynamically regulated by different cytokines during each hair cycle, probably mediated by multiple cell types. As our data indicated that tissue radiosensitivity is largely dependent on the state of somatic stem cells, the autonomous or non-autonomous factor(s) which critically determine the fate of McSCs under genomic stress needs to be defined to further understand the molecular mechanisms of tissue stress-resistance.

Figure 7. Maintenance of McSC pools under distinct types of genomic stress. (A) Schematic representation of the experimental design for the time course study. (B) Quantitative analysis of the number of unpigmented GFP+ cells in the Bg-sBg area of hair follicles treated with IR and/or 5FU. (C) Coat colors of mice treated with/without IR and 5FU. Shown are the coats of mice with the second hair after hair-cycle induction. (D) Schematic of different McSC states and their stress-resistance for self-renewal. McSCs are maintained in the Bg-sBg area throughout the hair cycle. McSCs are all in quiescent states (G0; blue) in hair follicles at telogen phase, but when hair follicles progress into anagen phase, approximately one-half of McSCs become activated to enter the non-G0 state (orange), self-renew in the niche and provide mature melanocytes to the hair bulb for hair pigmentation. These activated McSCs are Kit-dependent, chemosensitive and radioresistant. The coexistence of quiescent and activated stem cells in anagen hair follicles provides strong stress-resistance for maintenance of the stem cell pool to prevent their depletion and resultant hair graying. Data are presented as means ± SD; *P<0.01 as calculated by Student’s test.
It is notable that Kit expression with no melanin content is a good marker for McSCs in non-aged mice as we reported previously (Inomata et al., 2009). As shown in Figure 2C, specifically target cancer stem cells. McSCs in anagen hair follicles survive in a Kit-independent manner with a low but detectable level of Kit expression. However, Kit expression by McSCs at the G0 quiescent state becomes undetectable after a long quiescent state of the telogen phase in older mice (data not shown). The exact timing and the underlying mechanism(s) for that will be determined in future studies.

In quiescent stem cells, non-homologous end-joining (NHEJ), which has a lower fidelity for repairing DNA-duplex strand breaks than does homologous recombination (HR; Branzei and Foiani, 2008), is available and often causes genomic instability. These different DNA repair mechanisms may explain the higher radiosensitivity of quiescent McSCs, which are prematurely depleted upon their cell cycle reentry. Our data further indicate that two different activation states during anagen give more stress-resistance to the stem cell pools overall and prevent the induction of hair graying. As human scalp hair follicles, which have a long anagen phase and a short telogen phase, are more resistant to IR and sometimes become more pigmented after IR, the coexistence of quiescent and non-quiescent McSC populations in the niche may strengthen those hair follicle types to resist different kinds of stress.

Comparison of the radiosensitivity between immature stem cells and their differentiating progeny has been attempted. While it has been reported that stem cells are known to be more radiosensitive than their progenies (Barker et al., 2007; van der Meer et al., 1992; Milyavsky et al., 2010; Mohrin et al., 2010; Potten et al., 1997), it has been unclear whether cycling stem cells are really more radiosensitive than their progeny or not. We found that quiescent McSCs are radiosensitive and prematurely commit to differentiation without undergoing apoptosis in response to non-lethal genomic stress, while their amplifying progeny undergoes apoptosis in the same anagen hair follicles (data not shown). These results demonstrate that quiescent stem cells and their progeny disappear from the skin by taking different fates under genotoxic stress. This further suggests that the mechanism for IR-resistance of proliferating cancer stem cells might be common to that of renewing somatic stem cells. Future studies on the cellular and molecular mechanisms of the stress-resistance of somatic stem cells and cancer stem cells may be a key to devise cancer therapies which specifically target cancer stem cells.

**Methods**

**Animals**

*Dct-H2B-GFP* tg mice were generated in our laboratory. *Dct-lacZ* tg mice (gift from Ian Jackson, MRC; Mackenzie et al., 1997; Nishimura et al., 2002) and MITF-*ce* mice (gift from Lynn Lamoreux; Zimring et al., 1996) were crossed with *Dct-H2B-GFP* tg mice, as previously described (Inomata et al., 2009; Nishimura et al., 2005).

**Generation of Dct-H2B-GFP transgenic mice**

To generate melanocyte lineage-specific expression vectors for Dct-H2B-GFP, the three PCR-amplified fragments encoding Tyr-LCR, pDct-H2B-GFP-bGHpA, and pUC18 backbone including the Amp-resistant gene and replication origin were used. The linearized transgene excised from the *Escherichia coli*-derived plasmid sequence was injected into fertilized oocytes of C57BL/6J mice. Transgenic founders were identified by PCR analysis of tail DNA. Primers corresponding to GFP coding regions are as follows: 5′-TCGTAAGAACGACATTCGAG-3′ and 5′-GAACCTAGGTCAGCTTGC-3′. More details will be described elsewhere (Okamoto N et al., unpublished data).

**Hair cycle induction**

Seven-week-old C57BL/6 mice were anesthetized with diethylether and hair on their backs were depilated to synchronize anagen induction (Muller-Rover et al., 2001). Hair follicles progress into anagenl at 2 days after depilation and reach their maximal length (anagenV) at 9 days after depilation. Catagen-associated changes in hair follicles are seen from 18 to 20 days after depilation, and hair follicles return to telogen 25 to 30 days after depilation. To analyze the coat color change in the following hair cycles, hair depilation was performed every 30 days.

**Irradiation**

Mice were exposed to IR using RX-650 (Faxitron X-Ray, Tuscon, AZ, USA). Irradiation was carried out at a dose of 5 Gy at 100 kVp using two 0.5-mm aluminum filters for total skin irradiation. Irradiation was carried out by placing mice in a thin-walled plastic box.

**BrdU administration**

BrdU (Sigma, St. Louis, MO, USA; 2 mg/ml in PBS) was administered by a single intraperitoneal injection at different phases of the hair cycle (50 mg/kg body weight). To analyze the distribution of McSCs in S-phase in hair follicles, the skin was sampled 1 h after BrdU injection. For analysis of label-retaining McSCs, mice were treated with BrdU on day 2 or day 3 after depilation and then the skin was sampled at 1, 4, and 24 h after BrdU injection and on day 5 after the second depilation.

**ACK2 administration**

A rat monoclonal antibody against the c-kit receptor protein (ACK2) was prepared as previously described (Nishikawa et al., 1991; Nishimura et al., 2002). One milligram ACK2 was subcutaneously injected in the dorsal skin at days 1, 3, and 5 after depilation.

**5FU administration**

5-fluorouracil (5FU; Nacalai Tesque, Kyoto, Japan) was prepared in DMSO at 50 mg/ml and was topically administered to the dorsal skin (200 µl/mouse).

**Immunohistochemical analysis**

Immunohistochemical staining was performed as previously described (Inomata et al., 2009). Details of the methods and antibodies used are provided in the Supporting information.

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Supporting information

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

**Figure S1.** Cyclic coupling of radioresistance to hair graying with hair cycle progression.

**Figure S2.** Validation of Dct-H2B-GFP transgenic mice for stable identification of McSCs in hair follicles.
**Figure S3.** Depletion of Kit-dependent cycling McSCs from the anagen hair follicle niche by ACK2 treatment.

**Figure S4.** Chronological fate analysis of radiosensitive McSCs.

**Figure S5.** Schematic of the fate of McSCs and maintenance of the McSC pool after irradiation and/or ACK2 administration.

**Appendix S1.** Material and Methods.