Keratinocyte Stem Cells but Not Melanocyte Stem Cells Are the Primary Target for Radiation-Induced Hair Graying

Hitomi Aoki[1], Akira Hara[2], Tsutomu Motohashi[1] and Takahiro Kunisada[1]

Ionizing radiation (IR)–induced hair graying is caused by the ectopic differentiation of melanocyte stem cells (MSCs) in their niche located at the bulge region of the hair follicle. Keratinocyte stem cells (KSCs) in the bulge region are an important component of that niche. However, little is known about the relationship between MSC differentiation and the KSC niche during IR-induced hair graying. We found that both follicular MSCs and KSCs were affected by IR by using immunohistochemical detection of γH2AX as a genotoxicity marker. We also found that KSCs prepared from irradiated mice were functionally affected by IR as indicated by their reduced colony-forming activity in culture and the delayed hair cycle *in vivo*. However, these effects of IR on KSCs were temporal. The MSC population, which proliferated and differentiated to melanocytes, was persistently maintained after irradiation. In addition to the loss of colony-forming activity, irradiated keratinocytes including KSCs suppressed the colony formation of MSCs *in vitro*. Furthermore, pigmented hairs were not reconstituted *in vivo* in the presence of irradiated KSCs or keratinocytes. These results provide a previously unreported insight that the primary target of IR during the induction of hair graying is follicular KSCs rather than MSCs.

Journal of Investigative Dermatology (2013) **133,** 2143–2151; doi:10.1038/jid.2013.155; published online 9 May 2013

INTRODUCTION

Most adult tissues undergo constant cellular turnover called tissue homeostasis (Barker *et al.*, 2010; Voog and Jones, 2010). Stem cells ensure tissue homeostasis by continuously providing new cells to replace differentiated and/or damaged cells that are lost. In hair pigmentation, mature melanocytes are supplied from the melanocyte stem cell (MSC) population. MSCs, which are originally derived from the neural crest, were previously identified as immature Dct+/KIT^{low} melanoblasts located in the bulge area of hair follicles (Nishimura *et al.*, 2002, 2005). The MSCs directly adhere to hair follicle keratinocyte stem cells (HFKSCs), are maintained in that niche environment throughout the hair cycle, and self-renew only at early anagen to provide amplifying and differentiating progenies to the hair matrix for hair pigmentation (Nishimura *et al.*, 2002; Tanimura *et al.*, 2011). MSCs in the bulge region are preferentially induced to postmitotic ectopically pigmented melanocytes *in situ* upon reacting to various genetic deficiencies, genetic stresses, and the aging process, which eventually result in hair graying (Nishimura *et al.*, 2005; Inomata *et al.*, 2009; Aoki *et al.*, 2011a).

The stem cell microenvironment, or niche, is critical for stem cell maintenance (Scadden, 2006; Moore and Lemischka, 2006; Fuchs, 2009; Nishimura, 2011). The keratinocyte stem cells (KSCs) are responsible for the cyclic regeneration of hair follicles as well as a transient supply of progeny to the interfollicular epidermis and to sebaceous glands after wounding (Oshima *et al.*, 2001; Hsu *et al.*, 2011). HFKSCs are found in the bulge region, a distinct area of the outer root sheath at the lower permanent portion in mammalian hair follicles (Blanpain and Fuchs, 2006; Cotsarelis, 2006) as well as in the secondary hair germ (Greco *et al.*, 2009). KSCs in the bulge region and dermal papilla cells at the base of hair follicles are likely to be the niche for KSCs in the hair germ.

Ionizing radiation (IR) produces multiple clusters of double-strand breaks that mediate cell cycle arrest, apoptosis, and DNA repair (Riley *et al.*, 2008). In IR-exposed mouse epidermis, HFKSCs in the bulge region are resistant to DNA damage–induced apoptosis and do not undergo premature differentiation or cellular senescence. Higher expression of the antiapoptotic protein Bcl-2 (B-cell lymphoma 2) and the rapidly attenuated activation of p53 induce faster DNA repair activity in HFKSCs (Sotiropoulou *et al.*, 2010).

However, it has not been fully elucidated which of these two different stem cell populations in the bulge region of hair

[1]*Department of Tissue and Organ Development, Regeneration, and Advanced Medical Science, Gifu, Japan and* [2]*Department of Tumor Pathology, Gifu University Graduate School of Medicine, Gifu, Japan*

Correspondence: Takahiro Kunisada, Department of Tissue and Organ Development, Regeneration and Advanced Medical Science, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. E-mail: tkunisad@gifu-u.ac.jp

Abbreviations: HFKSC, hair follicle keratinocyte stem cell; IR, ionizing radiation; KSC, keratinocyte stem cell; MSC, melanocyte stem cell; Tg, transgenic

Received 20 November 2012; revised 27 February 2013; accepted 10 March 2013; accepted article preview online 2 April 2013; published online 9 May 2013

follicles is the primary target of IR-induced hair graying. In this study, we substantiated the specific deprivation of niche activity for melanocytes or MSCs from keratinocytes or KSCs following IR exposure, indicating that the primary target of IR during the induction of hair graying is HFKSCs rather than MSCs.

RESULTS

Not only MSCs but KSCs are also affected by IR exposure

IR-induced genotoxic stress was shown to induce unscheduled *in situ* differentiation of MSCs to pigmented melanocytes within 1 week, without showing any signs of apoptosis or senescence (Inomata *et al.*, 2009; Aoki *et al.*, 2011a). In post-IR mouse skin, we observed phosphorylated H2AX (γH2AX) immunoreactivity that is indicative of genotoxic effects on cells in the bulge region as well as the interfollicular epidermis and outer root sheath in hair follicles (Figure 1a and b and Supplementary Figure S1a–d online).

Those γH2AX-positive cells also expressed CD34, a marker for HFKSCs (Sotiropoulou *et al.*, 2010), as well as c-Kit, a marker for MSCs, as previously reported (Inomata *et al.*, 2009) (Figure 1c, d and Supplementary Figure S1e–x online). The number of γH2AX-positive HFKSC (Figure 1e) and the number of γH2AX-positive MSCs (Figure 1f) as well as their percentages (Supplementary Figure S1y online) and the number of γH2AX foci per nucleus (Supplementary Figure S1z online) are all clearly increased in post-IR mouse skin. Interestingly, the number of γH2AX-positive cells was significantly reduced in the epidermis of hk14-Kitl transgenic (Tg) mice in which Kitl is expressed in basal layer keratinocytes and confers a radioprotective effect on follicular MSCs (see Figure 5c–f in reference Aoki *et al.*, 2011a). As keratinocytes are not known to be affected by Kit signaling, the reduced number of γH2AX-positive cells in the bulge region was suspected to be MSCs that were influenced by KSCs expressing Kitl. These observations led us to consider the possibility of the IR-induced preferential destruction of the niche for MSCs in addition to MSCs themselves.

Delay of hair regeneration after IR exposure of the skin

To assess the effect of IR exposure on HFKSCs, adult mice plucked 1 day before exposure with or without 5 Gy IR were observed for their hair regeneration. Until 5 days after IR, no significant difference in the skins with or without IR was observed (Figure 1g and h). However, at approximately day 7 after IR, control mice showed blackly pigmented skin indicative of the anagen hair cycle, whereas irradiated mice did not have any pigmentation in their skin (Figure 1i). Irradiated mice began to show significant pigmentation at approximately day 10 (Figure 1j). Hair protrusion was apparent on day 12 in the control skin (Figure 1k) but was just recognizable on day 14 in the irradiated skin (Figure 1l). At day 17, the hairs of these two groups of mice grew in the same manner, although the irradiated skin developed gray hairs (Figure 1m). When newborn pups were irradiated, regeneration of the hair proceeded in a time course similar to the adult mice (Supplementary Figure S2a–i online). It is likely that the IR stress affects KSCs and prevents them from starting cell division for 3 days after plucking. However, after this latent time, KSCs start to proliferate normally to support the full restoration of hair even after severe IR that induces hair graying.

KSCs but not MSCs lose their colony-forming capacity after IR exposure of the skin: genotoxic stress imposed on KSCs may cause impaired niche function for MSCs

To investigate whether KSCs are impaired by IR exposure, we cultured cells prepared from the dorsal skins of newborn mice 6 hours to 5 days after irradiation. As follicular KSCs and MSCs of newborn skin were both affected as observed in the adult skin (compare Figure 1g–m with Supplementary Figure S2a–i online), we took advantage of newborn skin in which keratinocytes and melanocytes were both actively proliferating. The induction of γH2AX expression (Supplementary Figure S2j–l online) and the irregular differentiation of pigmented melanocytes (Supplementary Figure S2m–p online) possibly derived from MSCs were observed in the IR-exposed newborn skin, as similarly observed in the adult skin. TUNEL-positive cells were rarely detected in the IR-exposed newborn skin as well as in the nonirradiated skin (Supplementary Figure S2u and v online), and these TUNEL-positive cells were not significantly increased in the bulge area of the IR-exposed newborn skin (Supplementary Figure S2w online), indicating that apoptosis is not likely to be the major early fate of irradiated KSCs and MSCs.

At 2 weeks after cultivation in keratinocyte serum-free medium to allow keratinocyte expansion, cells from unirradiated dorsal skin formed characteristic keratinocyte colonies, whereas those exposed to 5 Gy IR produced fewer and smaller colonies when harvested 6 hours or 1 day after irradiation (Figure 2a and b). The colony-forming capacity then recovered by 2 days or later after IR exposure compared with that of the nonirradiated control (Figure 2a and b).

In contrast, when similar specimens were cultured in Dermalife to allow melanocyte expansion, the reduction of the number of melanocyte colonies was not so severe as that of KSCs even 6 hours or 1 day after irradiation (Figure 2c and d). At 2 days or later after IR, the colony-forming activities had recovered to the same level as the control (Figure 2c and d). The considerable reduction of melanocyte colonies at 5 days in irradiated skin-derived cells indicates the irreversible loss of MSCs from the hair follicle that corresponds to hair graying (Figure 2c and d).

This indicates that the genotoxic effect was manifested in KSCs for a short period after IR. In contrast, MSCs separated from KSCs defective of niche activity by IR were restored to form melanocyte colonies detected by DCT-LacZ-positive cells that mark melanocyte lineage cells (Mackenzie *et al.*, 1997), as shown in Figure 2e and f.

Our finding that KSCs are the first cell population to be affected by IR to reduce colony-forming activity suggests that the direct target of IR to induce gray hair is KSCs instead of MSCs. As a result, MSCs without the supportive niche may rapidly and irreversibly differentiate into mature melanocytes.