様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　５ 年　 ５ 月 ３１ 日現在

機関番号：１２１０２
研究種目：研究活動スタート支援
研究期間：2020～2022
課題番号：２０Ｋ２２６５９

研究課題名（和文）Defining the mechanism of epidermal stem cell heterogeneity in skin aging

研究課題名（英文）Defining the mechanism of epidermal stem cell heterogeneity in skin aging

研究代表者
Ｒａｊａ　Ｅｒｎａ（Raja, Erna）

筑波大学・生存ダイナミクス研究センター・助教

研究者番号：３０７７０５８１

交付決定額（研究期間全体）：（直接経費）　　2,200,000円

研究成果の概要（和文）：私達は、長期間にわたる系統追跡実験により、皮膚の老化において増殖周期の速い表皮幹細胞が経時的に枯渇していくのに対し、増殖周期の遅い表皮幹細胞は維持されていることを見出した。老化した皮膚では、表皮基底膜に局在する fibulin7の発現が減少しており、fibulin7欠損マウスでは、増殖周期の速い表皮幹細胞の消失やコラーゲンIV、コラーゲン XVII、ラミニンなどの基底膜関連タンパク質の変化、分化の異常など、皮膚の老化に似た特徴を示した。分子機構として、fibulin7は若年成人の皮膚における表皮幹細胞の増殖を遅くし、これが複製ストレスの蓄積と早期の幹細胞枯渇を最小限に抑えている可能性がある。

研究成果の学術的意義や社会的意義
私達は、表皮幹細胞の老化の特徴を明らかにし、皮膚における機能が知られていなかったfibulin 7が、どのように恒常性を維持し、増殖周期の遅い幹細胞集団と増殖周期の速い幹細胞集団のバランスを調節することで、老化のプロセスを制御するのか明らかにした。この知見は、皮膚の老化についての理解を深め、慢性創傷などの皮膚疾患の治癒に繋がると考えられる。

研究成果の概要（英文）：By long term lineage tracing, we found that the fast-cycling epidermal stem cells are depleted during chronological skin aging while the slow-cycling counterparts are maintained. Interestingly, fibulin 7 ECM expression which is localized to the epidermal basement membrane, is reduced in aged skin. Mice lacking fibulin 7 expression exhibited early skin aging-like traits such as loss of fast-cycling stem cells, alterations in basement membrane-associated proteins such as Collagen IV, laminins and Collagen XVII, and aberrant differentiation state. Mechanistically, fibulin 7 keeps a slower epidermal stem cell proliferation in the young adult skin and this may contribute to minimize the accumulation of replication stress and early stem cell depletion.

研究分野：skin biology

キーワード：skin aging　wound healing　regeneration　extracellular matrix　cell signalling　fibulin 7　stem cells heterogeneity

１．研究開始当初の背景

Tissue stem cells (SCs) are thought to be protected against aging by their slow cell division. SC aging may be contributed by repeated replication stress and accumulation of DNA damage (Behrens *et al*, 2014), which leads to metabolic and epigenetic alterations, aberrant proliferation and differentiation, and depletion of SC pools (Ermolaeva *et al*, 2018). It seems paradoxical that although the slow proliferation of SCs can minimize DNA damage, mutations acquired in slow-cycling SCs are more likely to accumulate over time due to the error-prone DNA repair pathway non-homologous end joining. This is in contrast to the homologous recombination pathway that is primarily adopted in faster-cycling SCs (Tumpel & Rudolph, 2019). Thus. whether the slow-cycling speed supports SC potential in the long run and delay stem cell aging in the skin remains unclear.

To study this, we utilized the slow- and fast-cycling SCs in the mouse skin epidermis as a model that enables us to compare the relationship between cell division frequency and aging within the same tissue environment. We have previously reported that slow-cycling (GFP label-retaining cells; LRC) and fast-cycling (non-GFP label-retaining cells; nLRC) populations were molecularly distinct and highly compartmentalized in the mouse skin epidermis (Sada *et al*, 2016). Slow-cycling SCs express *Dlx1,* while fast-cycling SCs express *Slc1a3*. The mouse tail skin serves as an excellent model of the slow- and fast-cycling SCs that undergo distinct differentiation programs to give rise to the keratin (K)10+ interscale and K31+ scale structures, respectively (Gomez *et al*, 2013; Sada *et al.*, 2016). These SC populations stay within their territorial boundaries in homeostasis but retain plasticity to contribute to each other's territory during injury repair (Sada *et al.*, 2016). However, it remains unknown how these two SC populations age and whether they are controlled by different molecular mechanisms. Here we report that aging led to disruption in the scale/interscale compartmentalization in the skin epidermis, along with gradual loss of fast-cycling SC clones and delayed wound healing. We further identified fibulin 7, a matricellular protein, as a regulator of the SC aging process that modulates long-term SC potential and extracellular matrix (ECM) maintenance.

２．研究の目的

We aimed to investigate if the slow-and fast-cycling SCs undergo distinct process of aging. Does faster proliferating SCs mean that they age faster? Furthermore, the ECM changes tremendously during aging. SC adhesion with the ECM is crucial in self-renewal and fate regulation, and alterations in the quality and quantity of ECM can induce aging-associated skin dysfunction (Egbert *et al*, 2014; Ge *et al.*, 2020; Koester *et al*, 2021; Liu *et al.*, 2019; Watanabe *et al*, 2017; Watt & Fujiwara, 2011). However, much is still unknown about the factors that regulate ECM changes in epidermal SCs during aging and it is still debated if skin aging occurs primarily due to SC-intrinsic factors or changes in the microenvironment (Ge *et al*, 2020). **We also wanted to evaluate the ECM changes and their roles in maintaining epidermal SCs heterogeneity to support homeostasis and skin regeneration over time.**

３．研究の方法

To examine stem cell's potential and behavior over time, we performed long-term lineage tracing using Dlx1-creER to label the slow-cycling SCs whereas fast-cycling SCs were labelled with Slc1a3-creER. Labelling were induced at postnatal day (PD) 49 with tamoxifen administration and skin samples were collected after 1-year-, 1.5-year- and 2-year-chase. Subsequently, to investigate transcriptomic changes in slow-and fast-cycling stem cells during aging we isolated these respective stem cell populations from the H2B-GFP tet-off mouse model (Tumbar *et al.*, 2004) from age PD49 compared to their 2-year-old counterparts and performed RNA-sequencing analysis.

To study the function of fibulin-7 we used *Fbln7* knock-out mouse model (Tsunezumi *et al.*, 2018) and analyse skin histology and immunostainings of proliferation marker, stem cell/differentiation markers and basement membrane proteins. We then crossed *Fbln7* knock-out mouse with Slc1a3-creER mouse and performed lineage tracing to test if fibulin 7 play a role in fast-cycling stem cells maintenance over time. We further tested skin regeneration ability by making full thickness wounding in the tail skin and follow up on the recovery over a month period. In-vitro cell culture-based experiments were also performed to assess the gain-of-function effects of *Fbln7* in primary newborn keratinocytes. Stable cells over-expressing *Fbln7* were tested for their proliferation and differentiation and their response to growth-stimulating interleukin such as interleukin-6. To gain insights on fibulin 7

mechanism, a screening assay for interacting proteins was performed using metal ion affinity chromatography and mass-spectrometry. Results were then validated using ELISA.

４．研究成果

Fast-cycling stem cells are gradually lost during aging

As previously observed (Sada *et al.*, 2016), from 1-week (1-w) to 1-year (1-y) post-labeling in homeostasis, Dlx1 slow-cycling clones were enriched in the interscale, whereas Slc1a3 fast-cycling clones were localized in the scale and interscale line regions. We found that the number and localization of Dlx1 clones were less affected during aging, but the number of Slc1a3 clones in the scale was significantly decreased, starting at a 1-y chase. It is noteworthy that while Slc1a3-labeled fast-cycling SC clones in the scale were gradually lost during aging, the small number of Slc1a3 clones in the interscale continued to thrive and expand within the interscale line and non-line regions. **Hence, fast-cycling SC clones were gradually depleted during aging, but slow-cycling SC lineages were maintained, suggesting that an imbalance in epidermal stem cell populations may occur in aged skin.** Along with changes at the SC level, wholemount images of K10 and K31 staining (the regional differentiation markers of interscale and scale, respectively) showed a significant age-dependent increase in the K10+ interscale area in 2-y-old mice compared with 2-month (2-m)-old mice. This result suggests that aging alters the unique lineage fate of epidermal SCs and disrupts their compartmentalization within the tissue.

Aging induces alterations in molecular properties of slow- and fast-cycling stem cells

To determine molecular factors that contribute to the loss of epidermal SC heterogeneity during aging, particularly the depletion of fast-cycling SCs, from our bulk RNA-seq analysis, we interrogated gene expression changes in 2-y-old slow-cycling SCs and fast-cycling SCs. Gene ontology (GO) analysis showed the 2-y slow-cycling SCs upregulated immune response and extracellular organization related genes. **Intriguingly, genes involved in DNA damage repair, telomere maintenance, DNA replication, and chromatin regulation were markedly reduced in old slow-cycling SCs, suggesting that aged slow-cycling epidermal SCs may be more prone to accumulating DNA damage.** To clarify this, immunostaining was performed with DNA oxidation marker 8-oxo-dG, which shows that cells detected with this marker were rare, although it is absent in the young skin. Further study is needed to determine whether there are differences in DNA damage repair mechanisms or the behavior of DNA-damaged cells between slow- and fast-cycling SC populations.

The aging fast-cycling SCs, on the other hand, undergo changes in genes related to cell metabolism, which is implicated in controlling SC proliferative heterogeneity and aging (Nakamura-Ishizu *et al*, 2020). To evaluate whether an imbalance between SC self-renewal and differentiation contributes to the age-related clonal decline in fast-cycling epidermal SCs, we examined changes in the expression patterns of established epidermal lineage marker genes (Ge *et al.*, 2020). We found that the expression of epidermal differentiation genes and HF-lineage markers, which were repressed in young fast-cycling SCs, were enhanced in older ones, suggesting that the maintenance of undifferentiated status and lineage identity may be compromised in aged fast-cycling epidermal SCs. **Hence, the RNA-seq results and lineage tracing analysis suggest that epidermal SCs with different division frequencies undergo distinct cellular and molecular processes and show functional decline with aging.**

Loss of *Fbln7* accelerates age-dependent depletion of fast-cycling stem cell clones and delays wound healing

Because both microenvironment and SC-intrinsic mechanisms govern the SC aging process, we focused on the secreted ECM genes of 2-y-old fast-cycling SCs, whose stem cell potential/lineage is compromised in aged skin. **Among the genes upregulated in the 2-y-old nLRCs with unknown function in the skin was fibulin 7 (gene symbol *Fbln7*), a secreted glycoprotein belonging to the short fibulin family of ECM proteins.** We observed fibulin 7 protein localization to the basement membrane (BM) in both the scale and interscale regions. Fibulin 7 expression was significantly lower in the *Fbln7* KO compared to wild-type (WT) controls and it was also significantly decreased in the 2-year-old tail skin of C57BL/6J (B6) WT mice compared to the 2-3-m-old counterparts. This suggests that although *Fbln7* mRNA was induced, fibulin 7 function may actually decrease in aged skin due to its lower protein abundance, in line with the observed loss of fast-cycling SCs in 2-y-old skin.

To test whether fibulin 7 modulates epidermal SC heterogeneity and behavior during aging, we labeled fast-cycling SCs with Slc1a3^{CreER} in the *Fbln7* KO background. Fast-cycling SC clones were localized in the scale region at 1-w or 3-m post-labeling, and some clones persisted for up to 1-y in *Fbln7* WT mice. Notably, at 1-w post labeling (in 2-m-old mice),

there was more proliferation observed in the *Fbln7* KO fast-cycling SCs as examined by Ki67 marker. In contrast, after 1 year, the number and size of fast-cycling SC clones in the scale region of *Fbln7* KO mice were significantly decreased compared with *Fbln7* WT mice. Hence, *Fbln7* exhibited an age-dependent function in the long-term maintenance of the fast-cycling SC population. It is probable that negative impacts on fast-cycling SCs at 1-y-old were linked to the earlier replication stress due to loss of fibulin 7 that induced more cell divisions at 2-3-m-old.

Slow- and fast-cycling SCs cooperate in response to skin damage (Sada *et al.*, 2016), and wound healing is delayed in aging skin (Keyes *et al.*, 2016; Liu *et al.*, 2019). We hypothesized that disturbed epidermal stem cell heterogeneity in the absence of *Fbln7* might reduce skin regenerative capacity upon injury. To test this, a wound-healing assay was performed in the tail skin of 2- to 3-m-old and 1-y-old mice in the presence or absence of *Fbln7*. While no apparent difference was observed in the wound healing ability of *Fbln7* WT or KO mice at 2–3-m of age, 1-y-old *Fbln7* KO mice showed impaired wound closure compared with *Fbln7* WT mice. Further examinations of the skin histology at the wound front indicate that the re-epithelialization process was hampered in 1-y-old skin lacking *Fbln7*, as shown by the newly formed epidermis that was shorter and thinner in the *Fbln7* KO mice. **Altogether, the expression of *Fbln7* in aging skin may minimize the loss of epidermal stem cell heterogeneity, supporting resilience against skin tissue damage.**

Fibulin 7 modulates stem cell inflammatory stress response and fate specification

To further understand how fibulin 7 supports long-term SC maintenance, RNA-seq was performed using basal epidermal SCs isolated from the dorsal skin of *Fbln7* WT and KO mice at 2-m or 1-y of age. The dorsal skin was utilized as it is the largest skin area containing the slow- and fast-cycling SC populations in the IFE (Sada *et al.*, 2016). PCA analysis showed that the transcriptomic difference between *Fbln7* WT and KO mice occurred at 1-y but not at 2-m of age, supporting our previous findings from lineage tracing and wound healing assays. Intriguingly, in the 1-y-old *Fbln7* KO mice, inflammatory response genes were upregulated, including antigen presentation, the MAPK cascade, and cytokine production while chemotaxis was both up- and downregulated. Lineage fate changes were reported as part of the inflammatory response (Ge *et al.*, 2017), and an analysis of these gene lists (Ge *et al.*, 2020) further underscored their alteration in 1-y-old *Fbln7* KO mice. The changes resemble some features of the 2-y-old nLRCs in the tail, such as enhanced hair lineage and IFE differentiation markers, albeit with some mouse-to-mouse variations.

Increased inflammation due to DNA damage has been linked to stem cell fate misspecification, which is manifested in the suprabasal expression of basal marker K14 (Seldin & Macara, 2020). We tested this in our model and found that unlike the K14 restriction to the basal layer in the 2-m-old B6 WT tail epidermis, K14 distribution significantly expanded from basal-to-suprabasal layers primarily in the scale region of the 2-y-old counterparts. Likewise, there was a significant increase in basal-to-suprabasal expansion of K14 in the scale region of the 1-y-old *Fbln7* KO mice but not in the 2-m-old *Fbln7* KO mice. Although suprabasal K14 was not observed in aged dorsal skin (Keyes *et al.*, 2016), it has been reported that aging promotes an inflammatory environment in skin (Doles *et al*, 2012; Hu *et al*, 2017); fast-cycling SCs in the tail scale may thus be more susceptible to this change. **Therefore, our results indicate that *Fbln7* loss increased the expression of inflammatory response genes in epidermal SCs, which may, in turn, influence the specification of SC fate.**

Fibulin 7 maintains the extracellular environment of epidermal stem cells by regulating basement membrane proteins

In the 1-y-old *Fbln7* KO mice, in addition to inflammatory response genes, ECM-related genes were also affected, such as BM components (collagen IV (col IV), nidogen 1, laminins, fibulin 1). To confirm this, immunostainings of col IV and laminins as major components of the BM were performed. Col IV staining was increased along the BM of 2-y-old versus 3-m-old B6 WT mice. A similar pattern was observed in the epidermis of 1-y-old *Fbln7* KO mice compared to WT. This aligns with a previous report describing the upregulation of col IV in thickened BM, which led to niche stiffness in aged skin (Koester *et al.*, 2021). Quantifications of BM col IV intensity demonstrated a significant increase in the scale and interscale of 2-y-old B6 WT; however, the increase was only significant in the scale of 1-y-old *Fbln7* KO epidermis and not in the interscale. On the contrary, laminin BM staining was unchanged in the 2-y-old B6 WT mice vs. 2-3-m-old mice, but its protein abundance was significantly lower in the *Fbln7* KO BM in both scale and interscale regions. Moreover, collagen XVII proteolysis promotes skin aging (Liu *et al.*, 2019), and we confirmed that collagen XVII staining was

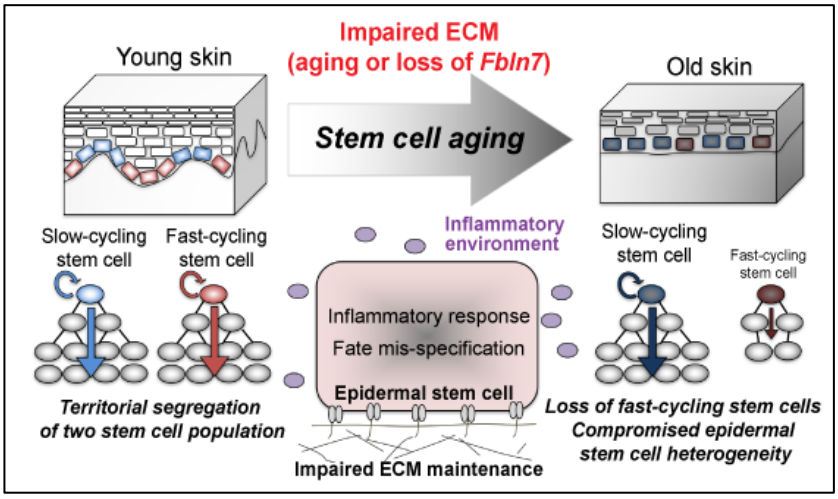
significantly decreased in the interscale region of 2-y-old B6 WT mice as well as in the scale and interscale regions of 1-y-old *Fbln7* KO mice. **These results suggest that the loss of fibulin 7 affects BM composition, favoring the aging-like conditions.**

To further characterize the biochemical functions of fibulin 7, we screened for secreted fibulin 7-binding proteins using conditioned media prepared from heparin-treated cells overexpressing full-length (FL) fibulin 7 or cells overexpressing fibulin 7 but lacking the N-terminal heparin-binding coiled-coil domain (dCC) (Tsunezumi *et al.*, 2018). The CC domain mediates binding to heparin and is important for the pericellular tethering of fibulin 7 (Tsunezumi *et al.*, 2018). The putative fibulin 7-binding proteins were co-eluted with fibulin 7 and the following classes of candidate proteins were selected: those with functions in the structural integrity of the BM; growth factors; matricellular proteins related to wound healing; and ECM remodeling proteins. We confirmed via solid-phase binding assay the dose-dependent interactions of fibulin 7 with BM components such as col IV and, to a lesser extent, the fibulin 1C and 1D isoforms. Fibulin 7 also exhibited direct binding to matricellular proteins that are upregulated upon wound healing or inflammation, such as tenascin C, periostin, and Ccdc80 (Hirota *et al*, 2012; Midwood *et al*, 2016; Nikoloudaki *et al*, 2020; Tremblay *et al*, 2009). **These results suggest that fibulin 7 physically interacts with ECM proteins that regulate BM composition and integrity, supporting the extracellular environment of epidermal SCs.**

Fibulin 7 regulates the undifferentiated state and proliferative response to growth factors in primary keratinocytes

In vivo phenotype analysis has so far suggested that fibulin 7 may function as a protective factor for epidermal SCs during physiological aging, maintaining their long-term stem cell potential. To investigate the function of fibulin 7 in primary mouse keratinocytes, we overexpressed the FL or dCC mutant. Overexpression of fibulin 7 suppressed differentiation markers (*Krt1*, *Krt10*, *Lor*, *Dsc1*), independently of its CC domain. The stem cell markers (*Krt14*, *Klf5*) and cobblestone-like morphology of keratinocytes were unchanged, suggesting that fibulin 7 maintains primary keratinocytes in an undifferentiated state. Fibulin 7 overexpression was able to inhibit differentiation markers (*Krt1* and *Krt10*) not only in the basal state but also during calcium induction in the presence or absence of col IV coating, albeit a more significant suppression was observed with col IV coating. Binding with col IV may enhance fibulin 7 function through their protein-protein interactions.

We further investigated the role of fibulin 7 in keratinocytes exposed to a high or low amount of serum growth factors, mimicking the aging skin microenvironment. The proliferation assays in high (15%) or low (3%) serum conditions demonstrated that fibulin 7 maintains a lower cell division frequency of keratinocytes and that this effect required the CC domain. GO analysis from 1-y-old *Fbln7* KO RNA-seq data indicated an upregulation of genes associated with interleukin-6 (IL-6) production. IL-6 induces hyperproliferation of keratinocytes in vitro and is associated with skin aging, wounding, and psoriasis (Barrientos *et al*, 2008; Doles *et al.*, 2012; Grossman *et al*, 1989; Hu *et al.*, 2017; Taniguchi *et al*, 2014). We found that responses to growth cues by this cytokine were blunted in fibulin 7-overexpressing keratinocytes. **Thus, fibulin 7 may be beneficial for suppressing differentiation and maintaining slower proliferation status in epidermal SCs.**



５．主な発表論文等

〔雑誌論文〕　計3件（うち査読付論文　2件／うち国際共著　0件／うちオープンアクセス　3件）

１．著者名 Erna Raja, Gopakumar Changarathil, Lalhaba Oinam, Yen Xuan Ngo, Jun Tsunezumi, Ryutaro Ishii, Takako Sasaki, Kyoko Imanaka-Yoshida, Hiromi Yanagisawa, Aiko Sada	４．巻 preprint
２．論文標題 The extracellular matrix fibulin 7 maintains epidermal stem cell heterogeneity during skin aging	５．発行年 2022年
３．雑誌名 BioRxiv	６．最初と最後の頁 preprint
掲載論文のDOI（デジタルオブジェクト識別子） 10.1101/2022.02.28.482241	査読の有無 無
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Lalhaba Oinam, Gopakumar Changarathil, Erna Raja, Yen Xuan Ngo, Hiroaki Tateno, Aiko Sada, Hiromi Yanagisawa	４．巻 19
２．論文標題 Glycome profiling by lectin microarray reveals dynamic glycan alterations during epidermal stem cell aging	５．発行年 2020年
３．雑誌名 Aging Cell	６．最初と最後の頁 e13190
掲載論文のDOI（デジタルオブジェクト識別子） 10.1111/acel.13190	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Raja E, Changarathil G, Oinam L, Tsunezumi J, Ngo YX, Ishii R, Sasaki T, Imanaka-Yoshida K, Yanagisawa H, Sada A.	４．巻 23 (12)
２．論文標題 The extracellular matrix fibulin 7 maintains epidermal stem cell heterogeneity during skin aging.	５．発行年 2022年
３．雑誌名 EMBO Reports	６．最初と最後の頁 e55478
掲載論文のDOI（デジタルオブジェクト識別子） 10.15252/embr.202255478	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

〔学会発表〕　計6件（うち招待講演　1件／うち国際学会　5件）

１．発表者名 Erna Raja
２．発表標題 The role of Fibulin 7 in the maintenance of mouse skin epidermal stem cells
３．学会等名 53rd Annual Meeting of the Japanese society for Matrix Biology and Medicine（国際学会）
４．発表年 2021年

１．発表者名
Erna Raja

２．発表標題
The role of Fibulin-7 in the maintenance of mouse skin epidermal stem cells

３．学会等名
The 94th Annual Meeting of the Japanese Biochemical Society（国際学会）

４．発表年
2021年

１．発表者名
Erna Raja

２．発表標題
Fibulin-7 maintains epidermal stem cells heterogeneity during skin aging

３．学会等名
The 44th Annual Meeting of the Molecular Biology Society of Japan（国際学会）

４．発表年
2021年

１．発表者名
Erna Raja

２．発表標題
Fibulin-7 maintains epidermal stem cells heterogeneity during skin aging

３．学会等名
Singapore International Skin Conference 2022（国際学会）

４．発表年
2022年

１．発表者名
Erna Raja

２．発表標題
Fibulin-7 maintains epidermal stem cells heterogeneity during skin aging

３．学会等名
Elastin and Related Molecular Research Meeting（招待講演）

４．発表年
2022年

１．発表者名 Erna Raja
２．発表標題 Fibulin-7 maintains epidermal stem cells heterogeneity during skin aging
３．学会等名 The 95th Annual Meeting of the Japanese Biochemical Society（国際学会）
４．発表年 2022年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

―

６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関