Rac-Dependent Signaling from Keratinocytes Promotes Differentiation of Intradermal White Adipocytes

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Rac signaling affects numerous downstream targets in vitro; however, few studies have established in vivo levels. We generated mice with a single knockout (KO) of Rac1 (Keratin5(KS)-Cre;Rac1floox/fox, Rac1-KO) and double KO of Rac1 and Rac3 (K5-Cre;Rac1floox/fox;Rac3−/−, Rac1/Rac3-DKO) in keratinocytes. The hairless phenotype in Rac1-KO mice was markedly exacerbated in Rac1/Rac3-DKO mice. Strikingly, Rac1-KO mice exhibited thinner dermal white adipose tissue, which was considerably further reduced in Rac1/Rac3-DKO mice. DNA microarray using primary keratinocytes from Rac1/Rac3-DKO mice exhibited decreased mRNA levels of Bmp2, Bmp5, Fgf20, Fgf21, Fgfbp1, and Pdgfz. Combinational treatment with bone morphogenetic protein (BMP) 2 and fibroblast growth factor (FGF) 21 in culture medium, but not individual purified recombinant proteins, could differentiate 3T3-L1 fibroblasts into adipocytes, as could culture media from primary keratinocytes. Conversely, addition of anti-BMP2 or anti-FGF21 antibodies into the culture medium inhibited fibroblast differentiation. In addition, BMP2 and FGF21 treatment promoted adipocyte differentiation only of rat primary white adipocyte precursors but not rat primary brown adipocyte precursors. Furthermore, BMP2 and FGF21 treatment enhanced adipogenesis of normal human dermal fibroblasts. Notably, brown adipogenesis promoted by FGF21 was inhibited by BMP2. Thus, we propose a complex paracrine pathway from keratinocytes to intradermal pre-adipocytes, which functions as a Rac-dependent modulator of both white and brown adipogenesis.

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INTRODUCTION

The skin, which is composed of three layers, the epidermis, the dermis, and subcutaneous tissue (Koster and Roop, 2007), functions as a barrier between the organism and the environment (Proksch et al., 2008). The physiological barrier of the epidermis is established during embryogenesis and mainly localized in the cornified layer. Keratinocytes constitute approximately 90% of epidermal cells (Nestle et al., 2009). The upper part of the dermis is divided into the papillary and reticular dermis, whereas the lower part contains white adipose tissue (WAT) (Driskell et al., 2013).

Two developmentally, morphologically, and physiologically distinct types of WAT have been proposed recently to exist in the skin: intradermal/dermal WAT (dWAT) beneath the reticular dermis and subcutaneous WAT, which are separated clearly by a striated muscle called the panniculus carnosus in rodents (Driskell et al., 2013). Immature adipocytes in the dermis promote hair follicle cycling through platelet-derived growth factor α (PDGFA) secretion (Festa et al., 2011), demonstrating the paracrine differentiation signaling from intradermal adipocytes to the stem cells in the follicular bulge. Additionally, the possibility of reciprocal signaling from hair follicle keratinocytes to intradermal pre-adipocytes is suspected (Donati et al., 2014; Jaboda and Christiano, 2011); however, the detailed mechanism remains unknown. Furthermore, adipose tissues are classified into both WAT and brown adipose tissue, with beige/brite adipocytes, comprising brown adipocytes induced in the WAT, proposed as a third adipocyte type (Sanchez-Gurmaches et al., 2016). Nevertheless, the different adipocyte induction mechanisms remain unclear.

Racs (Rac1–Rac3), the best characterized members of the Rho family of small GTPases, play fundamental roles in various cellular processes including transcriptional regulation and actin-based structure turnover (Namba et al., 2015; Ueyama and Small, 2019). Previously, the hairless phenotype was reported in mice with Rac1 deletion (Benitah et al., 2005; Castillo et al., 2007; Chrostek et al., 2006), wherein either the keratin 5 promoter (K5) or keratin 14 promoter was used to effect Rac1 deletion in keratinocytes. Rac1 loss in keratinocytes prevented epidermal stem cell renewal and caused robust commitment to stem cell differentiation, finally resulting in epidermal failure (Benitah et al., 2005).
K5-Cre generation of keratinocyte-specific Rac1/Rac3 crosstalk between epidermal keratinocytes and dWAT. Around postnatal day (P) 10 (P11 Rac3 c-KO mice) showed positivity in the interfollicular epidermis, hair follicle, and sebaceous gland (arrows) of K5-Cre/LacZ mice. d) Photographs of control, K5-Cre/Rac1flox/flox (Rac1−/−, Rac1-KO), and Rac1-KO with heterozygous Rac3-KO (Rac1−/−Rac3+/−) (P12, left and control, Rac1−/−Rac3−/−, and K5-Cre/Rac1flox/flox, Rac3−/− (Rac1−/−Rac3−/−). (d) K5-Cre;Rac1flox/flox:Rac3−/− (Rac1−/−Rac3−/−) mice (P11, right). K5-Cre;Rac1flox/flox:Rac3−/− mice exhibit strongest and exacerbated hairless phenotype. bp, base pairs; DKO, double knockout; K5, keratin 5 promoter; KO, knockout; M, marker; NC, negative control; P, postnatal day. (c) Bar = 300 μm. (d) Bar = 10 mm.

Additionally, the latter two groups reported the critical role of Rac1 in hair follicle development and maintenance. However, Rac3 function in keratinocytes and its synergistic (Nakamura et al., 2017; Vaghi et al., 2014) or antagonistic (Hajdo-Milasinović et al., 2007) interaction with Rac1 remain unknown.

To address these issues, we generated mice with Rac1 and Rac3 double knockout (DKO) in keratinocytes (K5-Cre;Rac1flox/flox:Rac3−/−, hereafter Rac1/Rac3-DKO). We revealed differentiation-inducing signaling from keratinocytes to intradermal adipocytes, confirming the existence of crosstalk between epidermal keratinocytes and dWAT.

RESULTS
Expression of Rac1/Rac3 in primary keratinocytes and generation of keratinocyte-specific Rac1/Rac3-DKO mice
Reverse transcriptase–PCR using primary mouse epidermal keratinocytes (PMEKs) obtained from wild-type mice identified Rac1 as the predominant Rac isoform expressed in PMEKS (Figure 1a), with lesser Rac3 expression (Figure 1b). In K5-Cre+/−;LacZ+/− mice, X-gal–positive cells, representing the pattern of K5-Cre–driven recombination, comprised keratinocytes in the interfollicular epidermis, hair follicles, and sebaceous glands (Figure 1c). Therefore, we successfully generated mice with Rac1-KO and Rac1/Rac3-DKO in keratinocytes by crossing K5-Cre with Rac1flox/flox or Rac1flox/flox;Rac3−/− mice (Figure 1d).

Rac1/Rac3-DKO mice exhibit thinner dWAT
Rac1-KO (Castilho et al., 2007; Chrostek et al., 2006) but not Rac3-KO mice (Rac3−/− and Rac1flox/flox;Rac3−/−) (Corbetta et al., 2005) exhibited a hairless phenotype (Figure 1d). Rac3-KO and Rac1flox/flox mice were subsequently used as controls. Around postnatal day (P) 10 (P11–12), Rac1-KO mice with Rac3 heterozygous deletion (K5-Cre;Rac1flox/flox;Rac3−/+ showed smaller body size than control (Rac1flox/flox and Rac1-KO mice, and an exacerbated hairless phenotype compared with Rac1-KO mice (Figure 1d). Rac1/Rac3-DKO hairless phenotype was exacerbated compared with that of K5-Cre;Rac1flox/flox;Rac3−/− mice, exhibiting relatively less black color (Figure 1d). Although all control (Rac1flox/flox, Rac3−/−, and Rac1flox/flox, Rac3−/−) and all except one Rac1-KO mice were alive at P30, K5-Cre;Rac1flox/flox, Rac3−/− mice exhibited a lower survival rate. Furthermore, all Rac1/Rac3-DKO mice died before P22 (before weaning) (Figure 2a). Rac1/Rac3-DKO mice exhibited significantly decreased body weight compared with Rac3-KO mice from P6 (Figure 2b).

To clarify the cause of early death of Rac1/Rac3-DKO mice, we first examined the barrier function of the skin. Control and Rac1-KO mice exhibited comparable toluidine blue permeability (Supplementary Figure S1a). Rac1/Rac3-DKO mice showed greater permeability than control (Rac3-KO and Rac3-KO plus heterozygous Rac1-KO) mice (Supplementary Figure S1b and c), albeit less than in a previous reported mouse line showing no lethality (Sokabe and Tominaga, 2010), indicating that permeability did not likely underlie the early death. We then examined cell types exhibiting a functional K5 promoter across the whole body. In K5-Cre+/−;LacZ+/− mice, X-gal staining was positive in sweat glands in plantar skin, epidermal cells in the brain, epithelial cells in the esophagus and stomach, and tracheal glands, but negative in the heart, spleen, liver, and kidney (Supplementary Figure S2). Thus, no obvious K5-mediated KO location could be attributed to Rac1/Rac3-DKO early death.

Histologic examination for Rac1/Rac3-DKO skin abnormality revealed significantly thin dermis of Rac1/Rac3-DKO mice compared with that of control (Rac1flox/flox) and Rac3-
KO mice from P0 (Figure 3a). Moreover, the Rac1-KO dermis became thinner than that of Rac3-KO mice at P3, and significantly thin compared with control and Rac3-KO dermis at P8 (Figure 3a). From P0, dermal thickness differed significantly between control-Rac1/KO and Rac1/Rac3-DKO mice (Figure 3a). Additionally, Rac1-KO and Rac1/Rac3-DKO mice presented hypertrophy in the epidermis and abnormal hair follicle morphology (Supplementary Figure S3).

To further examine the most severely affected dermal layer, we performed Oil Red O staining, which detects adipocytes. Rac1/Rac3-DKO mice exhibited significantly thinner dWAT than control and Rac3-KO mice from P0 (Figure 3b). Rac1-KO dWAT became significantly thinner than that of control and Rac3-KO mice at P8 (Figure 3b). These results are similar to the skin analysis findings (Figure 3a), suggesting that dWAT was the most severely affected dermal layer. Notably, the Rac1/Rac3-DKO dWAT was prevented from increasing its thickness from P0 (no significant difference between P0 vs P3 [P = 0.9992], P3 vs P8 [P = 0.9998], or P0 vs P8 [P = 0.8971] by two-way analysis of variance, Figure 3b). Moreover, Rac1-KO dWAT showed significant increase between P3 and P8 (P < 0.001, Figure 3b), but not P0 and P3 (P = 0.8413).

The upper portion of hair follicles number did not significantly differ among control, Rac3-KO, Rac1-KO, or Rac1/Rac3-DKO mice (Figure 3c). This was consistent with the reported limitation of hair follicle abnormalities to the non-permanent part of the hair follicle in Rac1-KO mice (Chrostek et al., 2006), which is not associated with cyclic hair follicle growth (Fuchs, 2007).

**Reduced secreted factors from Rac1/Rac3-DKO keratinocytes**

We hypothesized that the thinner dWAT in Rac1/Rac3-DKO mice is associated with decreased secreted factor(s) from Rac1/Rac3-DKO keratinocytes. To identify such secreted factor(s), we performed DNA microarray analysis using mRNAs obtained from PMEKs of control (Rac3-KO) and Rac1/Rac3-DKO mice (Figure 4a). Among the genes decreased >2-fold in the Rac1/Rac3-DKO relative to Rac3-KO keratinocytes (data are deposited in NCBI’s GEO, GSE122234), we selected six secreted factors as candidates: bone morphogenetic protein (BMP) 2, BMP5, fibroblast growth factor (FGF) 20, FGF21, FGFBP1, and PDGFz (Figure 4a). Bmp2, Fgf20, Fgf21, Fgfbp1, and Pdgfa mRNA reduction was confirmed by reverse transcriptase–PCR using equivalent samples (Figure 4b).

**3T3-L1 fibroblast differentiation to adipocytes by BMP2 + FGF21**

To examine the selected factor effects on adipogenesis, we first added purified recombinant BMP2, BMP5, FGF20, FGF21, and PDGFz proteins (purified recombinant FGFBP1 protein was not commercially available) into the 3T3-L1 fibroblast culture medium and examined adipogenesis by Oil Red O staining. 3T3-L1 cells supplemented with the five factors as well as those with dexamethasone, isobutylmethylxanthine, and insulin (hereafter DMI) as a positive control, but not cells with single factor addition, demonstrated positive Oil Red O staining (Figure 4c and d). To define the secreted factor(s) involved in 3T3-L1 cell adipogenesis, we analyzed the effect of all two-factor combinations (10 patterns). Three patterns, BMP2 + FGF21, BMP2 + FGF20, and PDGFz + FGF21, led to positive Oil Red O staining (Figure 4d). 3T3-L1 cell adipogenesis mediated by these three combinations was confirmed using immunoblotting for fatty acid binding protein 4 (FABP4), a marker of differentiated adipocytes (Figure 4d). Adipogenesis induced by BMP2 + FGF21 was also confirmed in C3H/10T1/2 mesenchymal cells, but was more difficult to achieve than that in 3T3-L1 cells (Supplementary Figure S4). Furthermore, extracellular signal-regulated kinase (Erk) 1/2 and c-Jun N-terminal kinase (JNK), but not p38 mitogen-activated protein kinase (MAPK), phosphorylation levels were reduced in Rac1/Rac3-DKO compared with those in Rac3-KO keratinocytes (Supplementary Figure S5).

**Induction of 3T3-L1 fibroblast adipogenesis via primary keratinocyte culture medium**

To confirm the effects of secreted factors from keratinocytes on adipogenesis, we used the culture medium of primary...
keratinocytes. HuMedia-KG2–based culture medium of PMEKs cultured for >48 hours (undiluted 48-hour–cultured PMEK medium, 2-fold–diluted 72-hour–cultured PMEK medium using fresh HuMedia-KG2 (Kurabo Industries, Osaka, Japan), and undiluted 72-hour–cultured PMEK medium) promoted increased FABP4 expression compared with that of fresh HuMedia-KG2, 24-hour–cultured PMEK medium, 2-fold–diluted 48-hour–cultured PMEK medium using fresh HuMedia-KG2, and DMEM containing 10% fetal bovine serum (Figure 5a). However, we were unable to examine the effects of Rac-KO PMEK culture medium because of the markedly decreased number of obtained PMEKs resulting from impaired adhesion and spreading on the culture dish, even in the case of Rac1-KO (Supplementary Figure S6), consistent with previous reports (Chrostek et al., 2006; Hamelers et al., 2005). Next, we examined the effects of HuMedia-KG2–based normal human epidermal keratinocyte culture medium, focusing on the endocrine-acting FGF21 rather than the paracrine-acting FGF20 (Goetz and Mohammadi, 2013). Increased FABP4 protein expression in 3T3-L1 cells was also observed in the undiluted 48-hour–cultured normal human epidermal keratinocyte medium, but was inhibited by adding anti-BMP2 antibodies in the culture medium (Figure 5b).
Furthermore, anti-FGF21 antibody addition ameliorated the increased FABP4 expression (Figure 5c). These results strongly supported the idea that secreted factors from keratinocytes, especially BMP2 + FGF21, promote dWAT adipogenesis.

**BMP2 + FGF21 induces white, but not brown, adipogenesis**

Thin dWAT in Rac1/Rac3-DKO mice and adipocyte differentiation of 3T3-L1 fibroblasts and C3H/10T1/2 mesenchymal cells suggested that BMP2 + FGF21 induces Rac-dependent adipogenesis. To assess which types of adipocytes are induced by BMP2 + FGF21, we used white and brown adipocyte precursor cells.

White adipocyte precursor treatment with BMP2 + FGF21 induced strong Oil Red O positivity (Figure 6a), in the order FGF21 < BMP2 < BMP2 + FGF21. This result was confirmed by FABP4 immunoblotting (Figure 6b). These findings supported that single BMP2 treatment may induce white adipogenesis, albeit not significantly; however, combinational treatment of BMP2 + FGF21 markedly enhanced white adipogenesis.

In sharp contrast, brown adipocyte precursor treatment with BMP2 + FGF21 did not induce increased levels of uncoupling protein 1 (UCP1), a brown adipocyte marker (Wang and Seale, 2016) (Figure 6c). Increased UCP1 protein levels were observed only in brown adipocyte precursors upon DMI or FGF21, but not BMP2 or BMP2 + FGF21, treatment. FGF21-mediated brown adipocyte induction is consistent with previous reports (Fisher et al., 2012). Notably, BMP2 addition (BMP2 + FGF21) inhibited FGF21-mediated UCP1 induction. Overall, combinational BMP2 + FGF21 treatment induced white, but not brown, adipogenesis, whereas BMP2 inhibited brown adipogenesis.

Finally, CD90-positive normal human dermal fibroblasts (NHDFs) derived from the reticular dermis, which reportedly differentiate into adipocytes (Driskell et al., 2013; Korosec et al., 2019), were used to verify the adipogenic potential of BMP2 + FGF21 toward primary fibroblasts. NHDFs were treated with or without BMP2 + FGF21, according to the established protocol to differentiate fibroblasts into adipocytes (Chen et al., 2017; Korosec et al., 2019; Scott et al., 2011). Although DMI or DMI with BMP2 + FGF21 afforded no adipogenesis, DMI + rosiglitazone and DMI + rosiglitazone with BMP2+FGF21 showed adipogenesis, as evaluated by FABP4 immunoblotting (Figure 6d). Notably, DMI + rosiglitazone-mediated adipogenesis was enhanced by BMP2 + FGF21 addition (Figure 6d). These results suggested that adipogenesis of NHDFs induced by BMP2 + FGF21 is more difficult than that observed in 3T3-L1, C3H/10T1/2, and adipocyte precursor cells, and that...
Several groups have reported the converse, from the presence of signaling from hair follicle keratinocytes to intradermal adipocytes to hair follicles (Chen et al., 2002; Festa et al., 2011; Jong et al., 1998). In mice, hair follicle downgrowth and morphogenesis are complete at P8 and approximately P16, respectively; this hair follicle developmental process with fully formed hair shafts is termed the “morphogenetic anagen” (Blanpain and Fuchs, 2006; Müller-Röver et al., 2001). Subsequently, the first hair follicle cycling is initiated after the catagen and telogen phases; the first actual growth phase (anagen) commences at 4 weeks after birth (Schneider et al., 2009). In our Rac1/Rac3-DKO mice, the skin failed to execute the final differentiation necessary to enter the hair growth cycle and increase the dWAT thickness. Although hair follicle atrophy in Rac1/Rac3-DKO mice may contribute to the thinner dWAT, these mice revealed the signaling from keratinocytes to intradermal pre-adipocytes, confirming the existence of reciprocal signaling and cross-talk between these cells.

BMPs, members of the transforming growth factor-β family, have been classified into several subgroups, including BMP2/4, BMP5/6/7/8, BMP9/10, and BMP12/13/14 (Katagiri and Watabe, 2016). These four subgroups activate BMP type I receptors along with SMAD1, 5, and 8 to regulate target gene transcription (Miyazono et al., 2010). BMP2 and BMP4 are generally reported to be involved in white adipogenesis, whereas BMP7 functions in brown adipogenesis (Cristancho and Lazar, 2011; Zhang et al., 2010). BMP4 also induces brown adipogenesis and browning of WAT (Elsen et al., 2011; Qian et al., 2013; Xue et al., 2014). Although BMP6 was reported to induce white adipogenesis (Donati et al., 2014), BMP6 was not identified as a downstream target of Rac in the present study. In addition, whereas Bmp2 and Bmp5 were detected as Rac1 downstream targets in keratinocytes, Bmp5 did not induce 3T3-L1 fibroblast adipogenesis even in combination with FGF21 or FGF20. Nevertheless, we confirmed that BMP signaling was coordinate with reduced phosphorylation levels of SMAD1/5 in the Rac1/Rac3-DKO dWAT (Supplementary Figure S7).

The mammalian FGF family comprises 22 members grouped into intracrine-acting, endocrine-acting, and five paracrine-acting subfamilies (Goetz and Mohammadi, 2013). FGF20 and FGF21 belong to the paracrine-acting and endocrine-acting subfamilies, respectively. FGF21 regulates glucose, carbohydrate, and lipid metabolism (Kurosu et al., 2007) and induces brown adipogenesis including browning in WAT (Dutchak et al., 2012; Fisher et al., 2012; Wei et al., 2012), whereas FGF20 is reportedly involved in hair follicle development (Huh et al., 2013). Periodic FGF21 and FGF20 expression in the skin, which depends on the hair growth cycle, has been reported (Kawano et al., 2005). As an endocrine-acting FGF, FGF21 does not appear to require FGFBPs. However, FGF20 is a paracrine-acting FGF; thus, FGFBPs may function in FGF20 delivery from keratinocytes to intradermal adipocytes. In the present study, we detected decreased Fgfbp1 mRNA levels in PMEKs; however, the binding capabilities of FGFBP1 to FGF20 and FGF21 are unknown.

Notably, constitutively active mutants of Rac1 and RhoG-DIβ, a Rho family small GTPase inhibitor, could promote and prevent BMP4-induced adipogenesis in C3H/10T1/2 cells, respectively (Huang et al., 2015). Additionally, signaling by
the canonical Wnt, Wnt/β-catenin, in the epidermis could promote adipogenesis along with epidermal development (Blanpain and Fuchs, 2006), in which BMP2 and BMP6 (Donati et al., 2014) and FGF20 (Huh et al., 2013) were secreted from keratinocytes and hair follicles following Wnt/β-catenin signaling activation, respectively. Furthermore, Rac1 involvement in Wnt/β-catenin signaling has been reported (Wu et al., 2008). Together, these reports and our present findings of reduced Bmp2 and Fgf20/Fgf21 mRNA levels in Rac1/Rac3-DKO keratinocytes support the possibility that Rac involvement in the Wnt/β-catenin signaling in keratinocytes promotes intradermal adipogenesis through BMP2 and FGF20/FGF21 secretion (Wnt/β-catenin → Rac → Bmp2 and Fgf20/Fgf21). Moreover, in support of the reduced phospho-Erk1/2 and phospho-JNK levels in Rac1/ Rac3-DKO keratinocytes (Supplementary Figure S5), phospho-Erk1/2 → BMP2 (Chen et al., 2010) and phospho-JNK → activating transcription factor 2 (ATF2) → FGF21 pathways were reported (Solinas and Becattini, 2017; Wang et al., 2017). BMP2 could also promote osteogenesis (Katagiri and Watabe, 2016), with low and high concentrations favoring adipogenesis and osteogenesis, respectively.
(Wang et al., 1993). Inhibition of Rac1 promoted BMP2-induced osteoblastic differentiation (Onishi et al., 2013). Alternatively, Rac inhibition was conversely reported to induce adipogenesis in 3T3-L1 cells (Liu et al., 2005). These reports suggest that Rac1 activity constitutes an important element for the commitment to adipogenesis, and that other factors, such as condition and cell type, may influence the adipogenesis process.

BMP2 and BMP4 secretion from keratinocytes has been reported (Jamora et al., 2003), along with BMP2 signaling from dermal adipocytes to the late anagen and early telogen hair follicles in adult mice (Plikus et al., 2008) and its adipogenic effects from the anagen hair follicles to dermal myofibroblasts during wound healing (Plikus et al., 2017). We found that BMP2 and FGF21 secreted from keratinocytes in a Rac-dependent manner induce white adipogenesis. We also demonstrated that FGF21, a brown adipogenesis inducer, facilitates white adipogenesis if applied in conjunction with BMP2. Furthermore, we identified a brown adipogenic inducer, Rac3, through the converse modulation of white and brown adipogenesis process.

MATERIALS AND METHODS

Animals

The Rac1\textsuperscript{floxed} (Ishii et al., 2017), Rac1\textsuperscript{floxed}/Rac3\textsuperscript{--/} (Nakamura et al., 2017), K5-Cre (Tarutani et al., 1997), and CAG-CAT\textsuperscript{floxed}-LacZ mice (Sakai and Miyazaki, 1997) were described previously. K5-Cre;Rac1\textsuperscript{floxed} and K5-Cre;Rac1\textsuperscript{floxed}/Rac3\textsuperscript{--/} mice were backcrossed to Rac1\textsuperscript{floxed} and Rac1\textsuperscript{floxed}/Rac3\textsuperscript{--/}, respectively, to generate experimental animals. This study was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulation (26-03-05). Details of animal experiments are provided in Supplementary Materials and Methods.

Histology

Section preparation, hematoxylin and eosin staining, and X-gal staining were performed as previously described (Ueyama et al., 2016; Ueyama et al., 2014; Ueyama et al., 2007). For Oil Red O staining, dorsal skins were dissected after transcardial perfusion with 0.9% saline solution. Sagittal cryosections, 3T3-L1 adipocytes, and rat primary adipocytes were fixed with 4% paraformaldehyde (15 minutes). After washing with 60% isopropanol, the fixed cryosections and cells were stained with Oil Red O solution (10 minutes) and photographed using a microscope equipped with a camera.

DNA microarray

DNA microarray was performed as previously described (Nakamura et al., 2017). Total RNA was extracted from P3 Rac3-KO and Rac1/ Rac3-DKO PMEKs. Gene expression profiles were examined using the SurePrint G3 Mouse GE 8 × 60K Microarray kit (Agilent Technologies, Santa Clara, CA).

Differentiation of 3T3-L1 fibroblasts to adipocytes by secreted factors or primary keratinocyte culture medium

3T3-L1 fibroblasts were cultured in 24-well plates using adipocyte growth medium (DMEM supplemented with MK425 (Takara Bio, Kusatsu, Japan)). Confluent 3T3-L1 cells were treated with the following reagents supplemented to the culture medium for 5 days: DMI as a positive control; 10 ng/ml of five human recombinant secreted factors (BMP2, BMP5, FGF20, FGF21, and PDGF-AAA (Wako Pure Chemicals, Osaka, Japan)); 10 ng/ml of ten combination patterns of two secreted factors; or 10 ng/ml of a single secreted factor.

PMEK and normal human epidermal keratinocyte culture media (HuMedia-KG2) were collected at 24, 28, and 72 hours after incubation, then centrifuged (5,000g). Confluent 3T3-L1 culture medium was exchanged with HuMedia-KG2 plus DMI, HuMedia-KG2, PMEK supernatants (24, 48, and 72 hours), PMEK supernatants diluted 2-fold using fresh HuMedia-KG2, HuMedia-KG2 with BMP2 + FGF21 or BMP2 + FGF20, or DMIEM with 10% fetal bovine serum, and incubated for 5 days. Alternatively, 3T3-L1 cells were cultured for 5 days using fresh HuMedia-KG2 or supernatants 48-hour cultured with normal human epidermal keratinocytes. Monoclonal antibodies against human BMP2 or FGF21 or control mouse IgG was added to 10 µg/ml final concentration in the culture medium.

Differentiation of rat primary white and brown pre-adipocytes to adipocytes using secreted factors

Rat primary white (Takara Bio) and brown (Cosmo Bio, Tokyo, Japan) pre-adipocytes were cultured in 24-well plates using adipocyte growth medium. At confluence, culture medium was changed into adipocyte growth medium supplemented with none (as a negative control) or 200 ng/ml BMP2, FGF21, or BMP2 + FGF21 and cultured for 10 days. Medium was changed once (at 5 days). As a positive control, DMI was added in the adipocyte growth medium for the first 3 days.

Differentiation of NHDFs to adipocytes by secreted factors

CD90-positive adult NHDFs (PromoCell, Heidelberg, Germany) were cultured in 24-well plates using fibroblast growth medium. Confluent cells were first precultured in adipocyte growth medium supplemented with or without 200 ng/ml BMP2 + FGF21 for 3 days; secondly in growth medium supplemented with DMI, BMP2 + FGF21, DMI + rosiglitazone (5 µM), or DMI + rosiglitazone + BMP2 + FGF21 for 3 days; and finally in adipocyte growth medium (without, with, without, or with BMP2 + FGF21, respectively) for 9 days (changed every 3 days).

Antibodies, reverse transcriptase–PCR, PMEK isolation, cell cultures, immunoblotting, and quantitative analysis and statistics

Details are provided in Supplementary Materials and Methods.

Data availability statement

DNA microarray data are available in the NCBI Gene Expression Omnibus (GEO) under accession number GSE122234.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.06.140

REFERENCES


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AUTHOR CONTRIBUTIONS

Conceptualization: TPey; Formal Analysis: TPey, MS, MN; Funding Acquisition: TPey, NS; Investigation: TPey, MS, MN, TPebi; Resources: AA; Supervision: NS; Validation: TPey; Visualization: TPey, MS, MN, TPeb; Writing - Original Draft Preparation: TPey; Writing - Reviewing and Editing: TPey, MS, MN, TPeb, TH, AA, NS.

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SUPPLEMENTARY MATERIALS AND METHODS

Antibodies and materials
The monoclonal antibodies against FABP4(D25B3)XP (#3544, RRID:AB_2278257, 1/1000), Erk1/2 (#9102, RRID:AB_330744, 1/1000), phospho-Erk1/2(Thr202/Thr204)(D13.14.4E)XP (#4370, RRID:AB_2315112, 1/2000), JNK(Thr183/Thr185)(81E11) (#4668, RRID:AB_823588, 1/1000), p38 MAPK (#9212, RRID:AB_330713, 1/1000), phospho-p38 MAPK(Thr180/Thr182)(D3Fp)XP (#4511, RRID:AB_330744, 1/500), Smad1/DK0 mice using SuperScript III reverse transcriptase — PCR
Reverse transcription was performed with 1 μg of total RNA obtained from keratinocytes of postnatal day (P) 1 control (Rac3) and Rac1/Rac3-DKO mice using SuperScript III reverse transcriptase and random primers (Invitrogen, Carlsbad, CA). The following primer pairs were used for PCR: 5′-GCA GAC AGA CGT GTT CTT AAT TTG C-3′ (forward) and 5′-TCA CAG CAG CCA TTT CCT CT-3′ (reverse, 358 base pairs [bp] predicted band) or 5′-TGG TAG GTG TGC GCC TAT-3′ (reverse, 455 bp) for Rac1, 5′-GGA GGA CTA TGA CCG CCT C-3′ (forward) and 5′-GGC CTT CTT CTT CTT G-3′ (reverse, 379 bp) for 5′-AAA TAG GAT GTG GCC TAT GAA CAT CC-3′ (forward, 382 bp) for Rac2, 5′-CCC ACA ACC ACC ACT TC-3′ (forward) and 5′-CAG TGC ACT TCC TGC TTC-3′ (reverse, 257 bp) or 5′-TGG AGC TAT ATC CCA AAA GGA G-3′ (reverse, 441 bp) for Rac3, 5′-CCT GAT GGA ATG GAT GAG ATC TA-3′ and 5′-TCA GGA CTA GGC ATG GCT GGC TCT G-3′ for Fgf21 (637 bp), 5′-CGT GAT GAG ACT CCA CAG CCT CC-3′ and 5′-TTA GCA TGA TGG CTT CAG TAA CAT G-3′ for Fgf21p (760 bp), 5′-CGG GAT GAG GAC CTG GGC TGG CC-3′ and 5′-TCA CCT CAC ATC TGT CTC CTC CTC CC-3′ for Pdgfa (569 bp), 5′-ACC ATG GTG GCC GGC ACC GC-3′ and 5′-ATA ACC ACA CCC GCA GGC CTC CAC CA-3′ for Bmp2 (1,188 bp), 5′-TCC ATG CTT CCG TGG ACC GAA G-3′ and 5′-TCA AGT GTA CAG TAG TGT TT G-3′ for Fgf20 (639 bp), and 5′-TGT TAC CAA CTG GGA CGA CA-3′ and 5′-TTT GAT GTC ACC GAG TT-3′ for beta-actin (415 bp).

Experiments using animals
All mice were identified by numbered ear tags. Mice were housed in specific pathogen-free conditions using an individually ventilated cage system (Techniplast, Tokyo, Japan), and allowed food and water ad libitum. The animal facility was maintained on a 14-hour light and 10-hour dark cycle at 23 ± 2 °C and 50 ± 10% humidity. Mice from the control group were always treated and assessed first, followed by the experimental group. Both males and females were used in the analyses because the phenotypes of Atoh1-Cre;Rac1flox/flox;Rac3-/- mice did not exhibit significant differences between the sexes. Mouse weight was monitored daily from P2 until the death of Rac1/Rac3-DKO mice. CAG-CATflox/LacZ mice were backcrossed with K5-Cre mice to generate K5-Cre+/+;LacZ+/+ mice. Experimental groups with mice older than 4 weeks were organized according to sex. The offspring of these mice were genotyped by PCR using the following primers: 5′-ACT CCT TCA TAA ACC CCT CG-3′ (forward) and 5′-ATC ACT CGT TGC ATC GAC CG-3′ (reverse) for K5-Cre, 5′-ATT TTC TAG ATT CCA GTT GTG AAC-3′ and 5′-ATC CCT ACT TCC TTC CAA CTC-3′ for Rac1flox, 5′-CAT TTC TGT GTC GCC GCC AAC-3′ and 5′-TGT CTG GTG TCC AGA AAT CAC-3′ for Rac3flox, 5′-CAT TTC TGT GGC GTC GCC AAC-3′ and 5′-CAG GCC GCC GAG CTG TTG TG-3′ for Rac3flox, and 5′-GCC TTA CCC AAC TTA ATC G-3′ and 5′-TGT GAG CCA GTA ACA ACC-3′ for LacZ. Wild-type C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan).

Primary mouse epidermal keratinocyte isolation and cell cultures
The mouse dorsal skins were rinsed with Hank’s balanced salt solution without calcium and magnesium (Wako Pure Chemicals, Osaka, Japan) and placed in 0.05% collagenase (Sigma-Aldrich, St. Louis, MO) overnight at 4 °C. The epidermis was peeled away from the dermis with forceps. Keratinocytes were detached from the epidermis by stirring and from the dermis by pipetting (Tsunenaga et al., 1994). After filtration using a 75 μm cell strainer (BD Biosciences, San Jose, CA), filtered cells were gathered by centrifugation at 750 g for 5 minutes and used for primary culture of keratinocytes, total RNA isolation, and immunoblotting. Primary mouse epidermal keratinocytes from P3 and normal human epidermal keratinocytes from neonatal foreskin (Kurabo Industries, Osaka, Japan) were plated on collagen-coated 6-well plates (Corning, Armonk, NY), and grown in HuMedia-KG2 supplemented with 0.03 mm Ca2+ and 0.1 ng/ml human epidermal growth factor, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.4% bovine pituitary extract, 50 μg/ml gentamycin, and 50 ng/ml amphotericin B (hereafter HuMedia-KG2, Kurabo) at 37 °C in 5% CO2, 3T3-L1 fibroblasts (RRID:CVCL_0123) and C3H/10T1/2 mesenchymal cells (RRID:CVCL_0190) were purchased from Japanese Collection of Research Bioresources Cell Bank (Ibaragi, Japan), and cultured in DMEM (Wako) containing 10% fetal bovine serum (Nichirei, Tokyo, Japan).
**Immunoblotting**
Cells were lysed in homogenizing buffer (Ueyama et al., 2007) by sonication in the presence of a protease inhibitor cocktail, a protein phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), and 1% Triton X-100. Total lysates were centrifuged at 800g for 5 minutes at 4 °C, then the supernatants were subjected to SDS-PAGE, followed by immunoblotting for 16 hours at 4 °C using primary antibodies diluted in phosphate buffered saline containing 0.03% Triton X-100. The bound primary antibodies were detected using secondary antibody—horseradish peroxidase conjugates using the ECL detection system (Bio-Rad Laboratories, Hercules, CA).

**Toluidine blue assay of newborn mice**
P0 mice were euthanized using carbon dioxide for 20 minutes. Mice were sequentially dehydrated in 25%, 50%, 75%, and 100% methanol for 1 minute each, and then immersed in 0.1% toluidine blue O (Chroma Gesellschaft Schmidt & Co., Münster-Roxel, Germany) in phosphate buffered saline (−) for 20 hours at 25 °C. After twice washing using phosphate buffered saline (−) for 20 minutes, mice were photographed.

**Differentiation of C3H/10T1/2 cells to adipocytes by secreted factors**
For differentiation of C3H/10T1/2 mesenchymal cells to adipocytes, confluent cells were first precultured in DMEM containing 10% fetal bovine serum with or without secreted factor(s) (50 ng/ml of BMP2, FGF21, or BMP2 + FGF21) for 6 days, secondly in culture media containing dexamethasone, isobutyl-methylxanthine, and insulin with or without the same secreted factor(s) for 3 days, and finally cultured for 6 days (changed every 3 days) with or without the same secreted factor(s).

**Quantitative analysis and statistics**
For quantitative analysis of Oil Red O staining, the number of Oil Red O-positive cells/field using a ×20 lens (10 fields per sample) were counted in plates and the thickness of intra-dermal adipose tissue was measured in sections.

For quantitative analysis of adipocytes, the number of cells with lipid drops/field as visualized using a ×20 lens (10 fields per sample) were counted in plates.

In DNA microarray, the background-subtracted signal intensity was subjected to 75th percentile normalization for inter-array comparison. The value was used as normalized signal intensity and the ratio of the normalized value of each gene (DKD/Rac3-KO) is presented.

For quantitative assessment of immunoreactive bands, protein expression levels were normalized to those of GAPDH using ImageJ software (National Institutes of Health, Bethesda, MD).

All data are presented as the means ± standard error of the mean. Two groups were compared using unpaired Student’s t-tests. For comparisons of more than two groups, one-way or two-way analysis of variance was performed followed by Tukey’s post hoc test of pairwise group differences. Statistical analyses were performed using Prism 6.0 software (GraphPad).

**REFERENCES**


Supplementary Figure S1. Barrier function in Rac1/Rac3-DKO mice.

Toluidine blue assay was performed at postnatal day 0 using (a) control (K5-Cre\(^{+/+}\);Rac1\(^{+/+}\) and K5-Cre\(^{+/+}\);Rac1\(^{+/+}\)) and K5-Cre\(^{+/+}\);Rac1\(^{+/+}\) (Rac1-KO) mice, and (b) control (K5-Cre\(^{+/+}\);Rac1\(^{+/+}\);Rac3\(^{+/+}\); K5-Cre\(^{+/+}\);Rac1\(^{+/+}\);Rac3\(^{+/+}\); Rac3\(^{+/+}\);Rac1\(^{+/+}\);Rac3\(^{+/+}\); Rac3\(^{+/+}\) (Rac1/Rac3-DKO) mice. (a) No difference in toluidine blue staining is observed between control and Rac1-KO mice. (b) Toluidine blue staining is stronger in Rac1/Rac3-DKO than in control mice. (c) Magnified dorsal skin photographs of Rac3-KO and Rac1/Rac3-DKO mice. Rac1/Rac3-DKO dorsal skin is more strongly stained by toluidine blue than Rac3-KO back skin. DKO, double knockout; K5, keratin 5 promoter; KO, knockout. (a and b) Bar = 10 mm. (c) Bar = 500 \(\text{\mu m}\).
Supplementary Figure S2. K5 promoter functioning cells, organs, and tissues detected by X-gal staining. K5-Cre−/−:LacZ−/− and K5-Cre+/−:LacZ+/− mice (a: P1, b-f: P25) were fixed by 4% paraformaldehyde, and then X-gal staining was performed using whole body, organs, tissues, and sections. (a) Whole bodies and (b) sections of plantar skins were subjected to X-gal staining. Whole skin, epidermis, and sweat glands (arrowheads) of K5-Cre+/−:LacZ+/−, but not K5-Cre−/−:LacZ−/−, mice are positive for X-gal staining. (c) Brains and sagittal sections including the ventricle were subjected to X-gal staining. Ependymal cells of K5-Cre+/−:LacZ+/−, but not K5-Cre−/−:LacZ−/−, mice are positive for X-gal staining. (d) Digestive tracts (from the esophagus to rectum) and sections of the esophagus and stomach were subjected to X-gal staining. Epithelial cells in the esophagus and stomach indicated by arrows in K5-Cre+/−:LacZ+/− mice are positive for X-gal staining. (e) Respiratory tract (from the trachea to the lung) and sections of trachea were subjected to X-gal staining. Tracheal glands (arrowheads) of K5-Cre+/−:LacZ+/−, but not K5-Cre−/−:LacZ−/−, mice are positive for X-gal staining. (f) The heart, spleen, liver, and kidney were subjected to X-gal staining. No apparent X-gal–positive signal is observed either in K5-Cre+/−:LacZ+/− or K5-Cre−/−:LacZ−/− mice. K5, keratin 5 promoter; P, postnatal day.
Supplementary Figure S4. Differentiation of C3H/10T1/2 mesenchymal cells to adipocytes by BMP2 + FGF21. C3H/10T1/2 cells were treated with DMI plus no factor, 50 ng/ml BMP2, FGF21, or BMP2 + FGF21. Fixed cells were stained using Oil Red O solution. Oil Red O staining was strongest in the BMP2 + FGF21 treated well. Lower panels are magnified images of the upper panels. Arrows indicate the Oil Red O-positive cells. Representative of at least three experiments. BMP, bone morphogenetic protein; DMI, dexamethasone, isobutyl-methylxanthine, and insulin; FGF, fibroblast growth factor. (upper) Bar = 5 mm. (lower) Bar = 250 μm.

Supplementary Figure S3. Morphology of the epidermis and hair follicles in Rac1/Rac3-DKO mice. Control, Rac3-KO, Rac1-KO (K5-Cre+/−;Rac1fl/fl), and Rac1/Rac3-DKO (K5-Cre+/−;Rac1fl/fl,Rac3−/−) mice (P8) were fixed with 4% paraformaldehyde, and then hematoxylin and eosin staining was performed using 10-μm dorsal skin sections. (a) Squares in the upper panels indicate the magnified areas shown in the lower panels. Rac1-KO and Rac1/Rac3-DKO mice show thicker epidermis (multiple layers of keratinocytes) compared with that of control and Rac3-KO mice. In addition, Rac1/Rac3-DKO mice exhibit a hypertrophic cornified layer. (b) Magnified images of areas indicated by squares in P8 panels of Figure 3a. (c) Magnified images of Rac1-KO and Rac1/Rac3-DKO hair follicles obtained from different mice. Arrowheads (in b and c) in Rac1-KO and Rac1/Rac3-DKO mice indicate hair follicles showing irregular and atrophic shape, which are more apparent in Rac1/Rac3-DKO mice. (d) Immunostaining using anti-cleaved caspase-3 antibody with eosin counterstaining. No apoptotic cells are detected in the hair follicles of control and Rac1/Rac3-DKO mice. DKO, double knockout; KO, knockout; P, postnatal day. Bar = 100 μm.

Supplementary Figure S5. Phosphorylation of Erk1/2, JNK, and p38 MAPK in Rac1/Rac3-DKO primary keratinocytes. Primary epidermal keratinocytes were obtained from Rac3-KO (control) and Rac1/Rac3-DKO mice. Lysates were subjected to SDS-PAGE followed by immunoblotting using anti-phospho-Erk1/2, JNK, and p38 MAPK antibodies. Phosphorylation of ERK1/2 and JNK, but not p38 MAPK, was reduced in Rac1/Rac3-DKO keratinocytes compared with that in Rac3-KO keratinocytes. Erk1/2, JNK, and p38 MAPK were used as loading controls. Representative of at least three experiments. DKO, double knockout; Erk, extracellular signal–regulated kinase; JNK, c-Jun N-terminal kinase; KO, knockout; MAPK, mitogen-activated protein kinase.
Supplementary Figure S6. Cell adhesion, cell spreading, and keratin 5-immunoreactivity in Rac1-KO keratinocytes. Primary epidermal keratinocytes were obtained from K5-Cre$^{-/-}$;Rac1$^{floxed}$/K0;Rac1$^{floxed}$ (control) and K5-Cre$^{+/-}$/K0;Rac1$^{floxed}$ (Rac1-KO) mice, and the same number of cells were cultured on collagen-coated plates or collagen-coated glass-bottom-dishes for 7 days. Phase contrast images were obtained using a microscope (Olympus CKX41) equipped with a camera (EOS Kiss X6i, Canon). Note the significantly impaired cell adhesion and spreading in Rac1-KO keratinocytes compared with control keratinocytes. Keratin 5-immunostaining (+DIC) images were obtained using a confocal laser microscope (LSM700, Zeiss). Note the significantly impaired cell spreading in keratin 5-positive Rac1-KO keratinocytes compared with control keratinocytes. Representative of at least three experiments. DIC, differential interference contrast; K5, keratin 5 promoter; KO, knockout. (left) Bar = 100 μm. (right) Bar = 20 μm.

Supplementary Figure S7. Phosphorylation of Smad1/5 in Rac1/Rac3-DKO dWAT. dWAT was obtained from Rac3-KO (control) and Rac1/Rac3-DKO mice. Lysates were subjected to SDS-PAGE followed by immunoblotting using an anti-phospho-Smad1/5 antibody. Phosphorylation of Smad1/5 was reduced in dWAT of Rac1/Rac3-DKO mice compared with that of Rac3-KO mice. Smad1 was used as a loading control. Representative of three experiments. DKO, double knockout; dWAT, intradermal white adipose tissue; KO, knockout.