



Reply to Chi et al.

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TO THE EDITOR

We are grateful to Bruce Morgan and his colleagues for engaging in a dialog over our own article (Kaushal et al., 2015). We believe that open discussion about conflicting observations is important in moving forward research—far too often results that are at odds with one another are simply ignored.

There are certainly aspects of the Chi et al. (2017) study that are superior to ours. In particular, we agree that there are advantages of studying the dermal papilla (DP) in unpigmented skin, and we note that the quantitation by Chi et al. is based on a larger number of hair follicles, and mice, than in our study.

Chi et al. (2017) suggest that our results are due to misidentification of hair follicle types. We agree that DP size alone cannot be used to identify hair follicle type. We analyzed skin of P65 mice in which the hair follicle cycle was asynchronous so that we could report on anagen (stage IV; early anagen of the second hair follicle cycle, according to the classification of Müller-Röver et al. [2001]) and telogen follicles from the same mice. We believe that Chi et al. are looking at a later anagen stage. Given the low frequency of non-zigzag hairs in our samples, it is unlikely that they would skew our data.

The tissue in our article (Kaushal et al., 2015) was not flash frozen but fixed with 4% paraformaldehyde before being cryopreserved in Optimal Cutting Temperature medium. Tissue preservation under these conditions is excellent (Driskell et al., 2012, 2013). The movies in the Supplemental Material of Kaushal et al. show that the 60- μm —thick horizontal whole mounts

are sufficient to capture all of the cells within a DP.

Several differences in the experimental approaches could contribute to the different results:

1. Chi et al. (2017) use a constitutively expressed Cre recombinase (Cre), not a tamoxifen inducible Cre (CreER), for genetic labeling and thus do not obtain temporal control of Cre activity. Further, no control for efficient recombination/ β -catenin stabilization using this Cre line is provided in the current or previous (Enshell-Seijffers et al., 2010) study.
2. In the 2010 *Developmental Cell* article by Enshell-Seijffers et al., the researchers describe Corin-Cre activity as being first detected at P3 in some DP cells; however, Corin is not homogeneously expressed in all DP cells until P7. In contrast, Kaushal et al. (2015) treated mice with tamoxifen to induce CreER at P1 and P2, before Corin-Cre is active. The use of different promoters to drive Cre expression and the activation of Cre at different times prevents direct comparison of the results.
3. Having worked with a number of different reporter lines, in our opinion the Rosa26-tdTomato reporter mouse line of Kaushal et al. (2015) is superior to the Rosa26-YFP reporter used by Chi et al. (2017), in terms of both recombination sensitivity and fluorescence expression level.

Finally, two recent articles by Zhou et al. (2016a, 2016b) support our conclusions (Kaushal et al., 2015). These researchers found that “expression of ΔN - β -catenin in CD133⁺ DP cells leads

to increased DP cell proliferation” (p. 12) and that “in line with this finding, the number of DP cells was increased in mutant hair follicles. . . . Analysis of skin histology showed that the mean size of DPs in mutant CD133-CreERT2; Rosa-rtTA; tetO-Ctnnb1 ΔN hair follicles . . . was increased compared with controls during early anagen stages” (p. 14) (Zhou et al., 2016a). These researchers have further reported that expression of a stabilized form of β -catenin promotes clonal growth of CD133⁺ DP cells in an in vitro three-dimensional hydrogel culture and on transplantation promoted in vivo hair growth in reconstituted skin compared with control cells (Zhou et al., 2016b).

In closing, we wish to thank you once again for the opportunity to discuss our results and look forward to further important contributions from the Morgan lab on the regulation and function of the dermal papilla.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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Abbreviation: DP, dermal papilla

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Decreased Filaggrin Level May Lead to Sweat Duct Obstruction in Filaggrin Mutant Mice

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TO THE EDITOR

Although sweat is considered to be one of the major aggravating factors of atopic dermatitis (AD), recent studies have indicated the beneficial effects of sweat on host defense and anti-inflammatory mechanisms (Murota et al., 2015; Watabe et al., 2013). Several studies have demonstrated that sweating is attenuated in patients with AD (Matsui et al., 2014a, 2014b), but the precise underlying mechanisms remain unclear.

Filaggrin (FLG) is a key protein involved in skin barrier function that is known to be decreased in the epidermis of patients with AD (Otsuka et al., 2014, Palmer et al., 2006). FLG contributes to stratum corneum (SC) hydration and skin pH. FLG also plays an important role in keratinocyte integrity, because a decreased FLG level gives rise to SC disintegration and abnormal keratinocyte differentiation (Brown and McLean, 2012; Pendaries et al., 2014). We consider that a decreased FLG expression in keratinocytes may affect the structure and function of sweat ducts that go through keratinization, which may eventually lead to a decrease of sweat secretion.

To examine this hypothesis, we performed an iodine starch test, which involves the subcutaneous injection of acetylcholine into the footpads of wild-type (C57BL/6N (B6)) and *Flg* mutant mice (for further details of our materials and methods, see [Supplementary Materials and Methods](#) online, and the study protocol was approved by the Animal Experimentation Committee of Kyoto University). Of note, the *Flg* mutant mice harbor only an *Flg* mutation that was segregated from flaky tail mice (double-homozygous *Flg* and *matted* [*ma*] mutations), and were backcrossed more than 10 times with wild-type B6 mice. The number of sweat spots (represented by black dots) was significantly decreased in *Flg* mutant mice compared with wild-type mice (Figure 1a and b). In addition, SC conductance, which is an indicator of SC hydration, after subcutaneous acetylcholine injection, gradually increased in nonanesthetized mice. The increment of SC conductance of the *Flg* mutant mice was significantly lower compared with that of the wild-type B6 mice (Figure 1c). We confirmed that the skin conductance level from the abdomen, where free of sweat glands,

exhibited a significantly lower skin conductance compared with that of the footpads (Supplementary Figure S1 online). Next, to confirm that sweating is decreased in *Flg* mutant mice, we analyzed the water content and main chemical compositions of the skin at various depths using in vivo confocal Raman spectroscopy as reported previously (Amano et al., 2015). After subcutaneous acetylcholine injection, the percentage change from the baseline for water content was significantly higher in wild-type mice compared with *Flg* mutant mice (Figure 1d). Interestingly, lactate, which is abundant in sweat (Watabe et al., 2013), was also significantly decreased near the skin surface in *Flg* mutant mice compared with wild-type mice (Figure 1e). These findings suggest that *Flg* mutation causes the decrease in sweating in the murine model.

Next, we histologically evaluated the acrosyringia, the intraepidermal part of the sweat duct, using a standard hematoxylin and eosin staining. The number of obstructed acrosyringia was pronouncedly higher in *Flg* mutant mice compared with wild-type mice (Figure 2a and b). To evaluate the nature of the obstructed material seen in the sections, we performed immunofluorescence staining using an antibody against mouse keratin 6 that is expressed only in the sweat ducts and not in the eccrine glands (Taylor et al., 2012). Interestingly,

Abbreviations: AD, atopic dermatitis; FLG, filaggrin; SC, stratum corneum

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