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Letter to the Editor

Overexpression of alkaline phosphatase improves the hair-inductive capacity of cultured human dermal papilla spheres

To the Editor,

The dermal papilla (DP) regulates the overlying epithelial cells and plays a key role in the regulation of hair growth and regeneration. However, unlike the intact DP, two-dimensional (2D)-cultured DP cells lack hair-inductive capacity (trichogenicity) [1,2]. Recently, several studies have shown that the cultivation of DP cells as three-dimensional (3D) spheroids can restore their trichogenicity [3–5].

The pilosebaceous units express alkaline phosphatase (ALP) liver/bone/kidney, or ALPL. ALP activity is a useful marker for identifying the location, shape, and size of DP [6,7], and it is correlated with the trichogenicity of DP cells [7]. Furthermore, ALP activity and *ALPL* gene expression are restored in DP cells with restored trichogenicity and in DP cell cultures that exhibit the intrinsic properties of DP [8–10].

In this study, we investigated whether the restoration of ALPL expression observed in DP spheres plays a critical role in their trichogenicity. Furthermore, we demonstrated how ALPL regulates the hair-inductive capacity of DP cells. We initially investigated whether inducing sphere formation in interfollicular dermal fibroblast (DF) cells restores ALPL expression. We observed that ALP activity was minimally restored in DF spheres, whereas it was fully restored in DP spheres (Fig. S1). In addition, we observed that DF spheres, in combination with fresh neonatal mouse epidermal cells, failed to induce hair follicles (Fig. S2). These initial findings prompted us to investigate the functional role of ALPL in the trichogenicity of human DP cells.

We overexpressed *ALPL* in DP spheres and carried out a hair reconstitution assay. DP cells (passage 2-3) from hair follicles dissected from non-balding scalps were used for the assay. A control vector or the ALPL overexpression vector was transfected into 2D-cultured DP cells for 48 h. The transfected cells were then reseeded on HydroCell plates for sphere formation. Subsequently, the DP spheres were mixed with neonatal mouse epidermal cells and implanted into nude mice (Fig. 1a). *ALPL* transcript levels in the overexpression vector-transfected DP spheres, although highly variable, were significantly higher than those in the control vector-transfected DP spheres (Fig. 1b). ALP activity was also markedly augmented in the ALPL vector-transfected spheres (Fig. 1c). Overexpression of ALPL significantly increased hair follicle induction (Fig. 1d). The ALPL vector-transfected DP spheres produced 43.3 ± 12.3 hair follicles, whereas the control vector-transfected DP spheres produced 13.5 ± 8.8 hair follicles (Fig. 1e). The numbers of induced hair follicles varied among donors of the DP lines. However, overexpression of ALPL increased trichogenicity of DP spheres regardless of DP cell preparation. We also observed that more hairs were induced in DP spheres with higher ALPL levels when we transfected varying amounts of an ALPL vector into a DP line, demonstrating that ALP activity of DP sphere is important for the hair induction (Fig. S3). Although the increased ALP activity in DP spheres augmented the efficiency of the number of forming reconstituted follicles, individual follicles possessed similar amount of ALP activity (Fig. S4).

To confirm the human origin of the DP cells from reconstituted hair follicles, the cells were labeled with a fluorescent dye, DiI, before sphere formation. Consistent with our previous findings [4], all DPs from reconstituted hair follicles harbored DiI-positive cells (Fig. S5). However, the possibility of mouse cell contamination from host nude mice or with mouse DP cells from fresh neonatal mouse epidermal cells in reconstituted DP cannot be excluded. Consistently, it was observed that certain portions of DPs harbored cells with a weak DiI signal or DiI-negative cells.

To further elucidate the ALPL-mediated hair-inductive capacity of DP spheres, we examined the effect of ALPL on the Wnt/β-catenin signaling pathway, one of the DP signature pathways known to maintain trichogenicity in DP cells [10]. Initially, we measured the levels of Wnt/β-catenin signaling proteins and pTopflash (a β-catenin-responsive TCF reporter) activity in DP spheres. Immunoblot analysis showed that ALPL overexpression increased the levels of phosphorylated AKT (p-AKT), leading to increased levels of phosphorylated GSK3β (p-GSK3β), which in turn resulted in increased β-catenin levels in the nucleus (Fig. 2a). Consistently, ALPL overexpression significantly increased pTopflash activity (Fig. 2b). To further confirm the regulation of Wnt/β-catenin signaling by ALPL, we examined whether the expression levels of known target genes of the Wnt/β-catenin signaling pathway were changed by altering ALPL expression levels. The expression levels of Wnt/β-catenin target genes, such as *Axin2*, *VCAN*, and *Lef1*, were significantly increased by ALPL overexpression (Fig. 2c). In addition, we observed that the expression level of *BMP4* changed significantly, whereas the expression levels of *BMP2*, *NOG*, and *FGF7* did not change significantly by ALPL overexpression (Fig. 2c). The expression levels of known DP signature genes [8,10] such as *ID2*, *Sox2*, and *Hey1* were also significantly increased upon ALPL overexpression (Fig. 2c).

In this study, we employed expression vector-mediated *ALPL* overexpression in combination with a hair reconstitution assay to elucidate the functional role of ALPL in the hair-inductive capacity of human DP spheres. We did not observe altered ability of sphere

Abbreviations: DP, dermal papilla; 2D, two-dimensional; 3D, three-dimensional.

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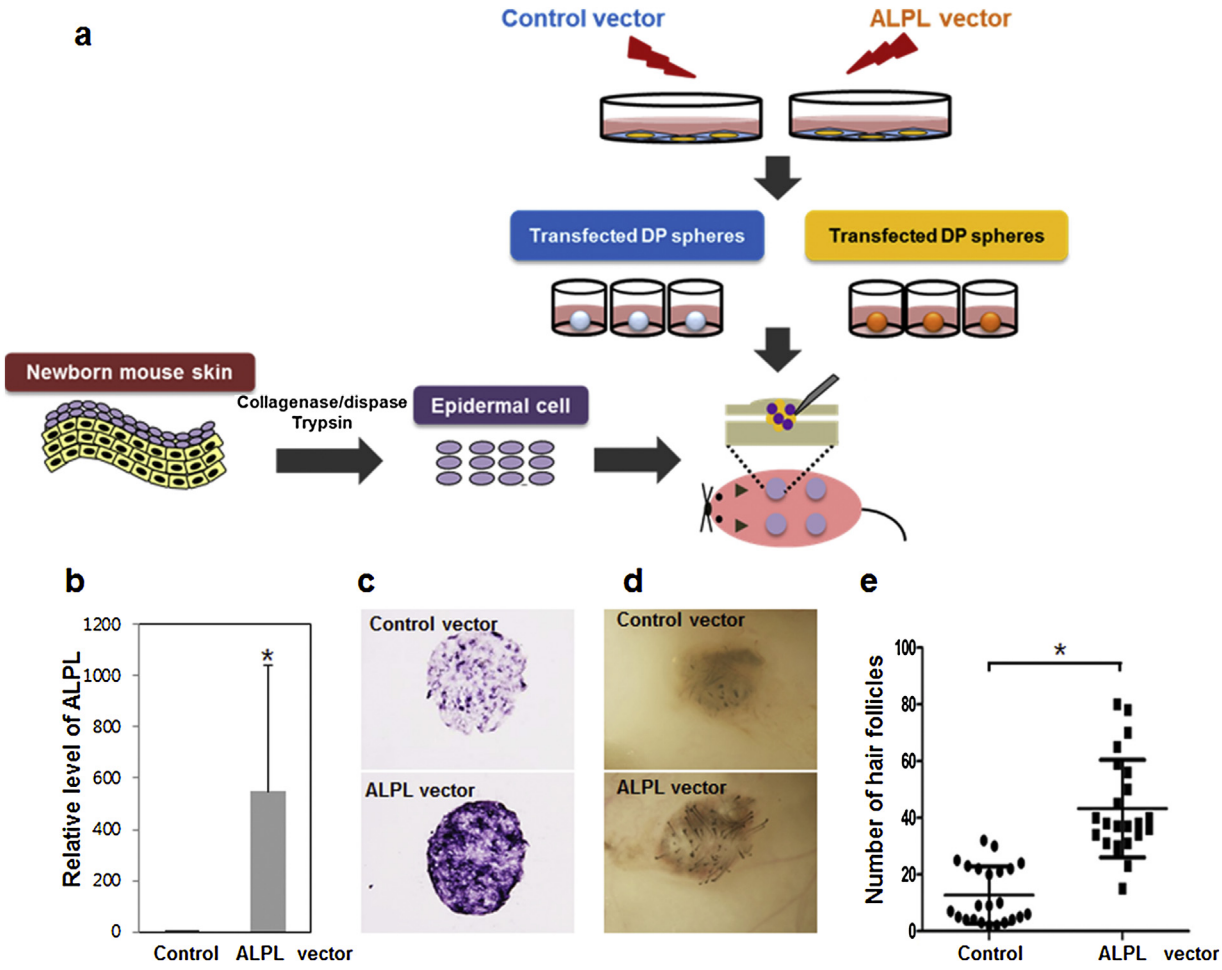

Fig. 1. Overexpression of *ALPL* augments the hair-inductive capacity of human DP spheres. (a) Diagram of the experimental procedure. (b) Relative levels of ALPL in ALPL expression vector-transfected spheres or control vector-transfected spheres were measured by real-time PCR. (c) DP cells were transfected with *ALPL* expression vector for 48 h, and the ALP activity was assessed 48 h after sphere formation. (d) A total of 100 DP spheres were co-implanted with mouse epidermal cells (10^6) into nude mice, and the injection sites were harvested 17 days later. (e) Data are expressed as the mean ± SD of the number of induced hair follicles at each injection site from six independent experiments using six different cell lines (n = 23, $^*P < 0.05$).

formation by overexpression of *ALPL* (Figure S6). Overexpression of *ALPL* was verified by enzyme activity staining and real-time PCR. Overexpression of ALPL augmented the hair-inductive capacity of human DP spheres (Fig. 1). Our data are in agreement with the finding that restoration of hair-inductivity by supplementation with various factors or by sphere formation is always accompanied by restoration of ALPL expression in DP cells [5,9,10]. We also found that overexpression of ALPL in DP spheres dramatically increased nuclear β-catenin levels, pTopflash activity, and the expression of target genes in the Wnt/β-catenin pathway (Fig. 2). Based on these results, we propose that ALPL regulates Wnt/β-catenin signaling in DP spheres. Since some of the DP signature genes are regulated by ALPL expression (Fig. 2), we hypothesize that ALPL is also involved in maintaining the *in vivo* status of the DP.

In conclusion, we report that ALPL augments the hair-inductive capacity of human DP spheres by regulating the Wnt/β-catenin signaling pathway and by maintaining the characteristics of the DP. Since competent dermal cells are necessary for the cell-based treatment of hair loss, our finding of ALPL involvement in hair-inductive capacity of human DP spheres will provide a rationale for a new strategy for preparing competent DP cells by augmentation of ALPL expression in DP spheres.

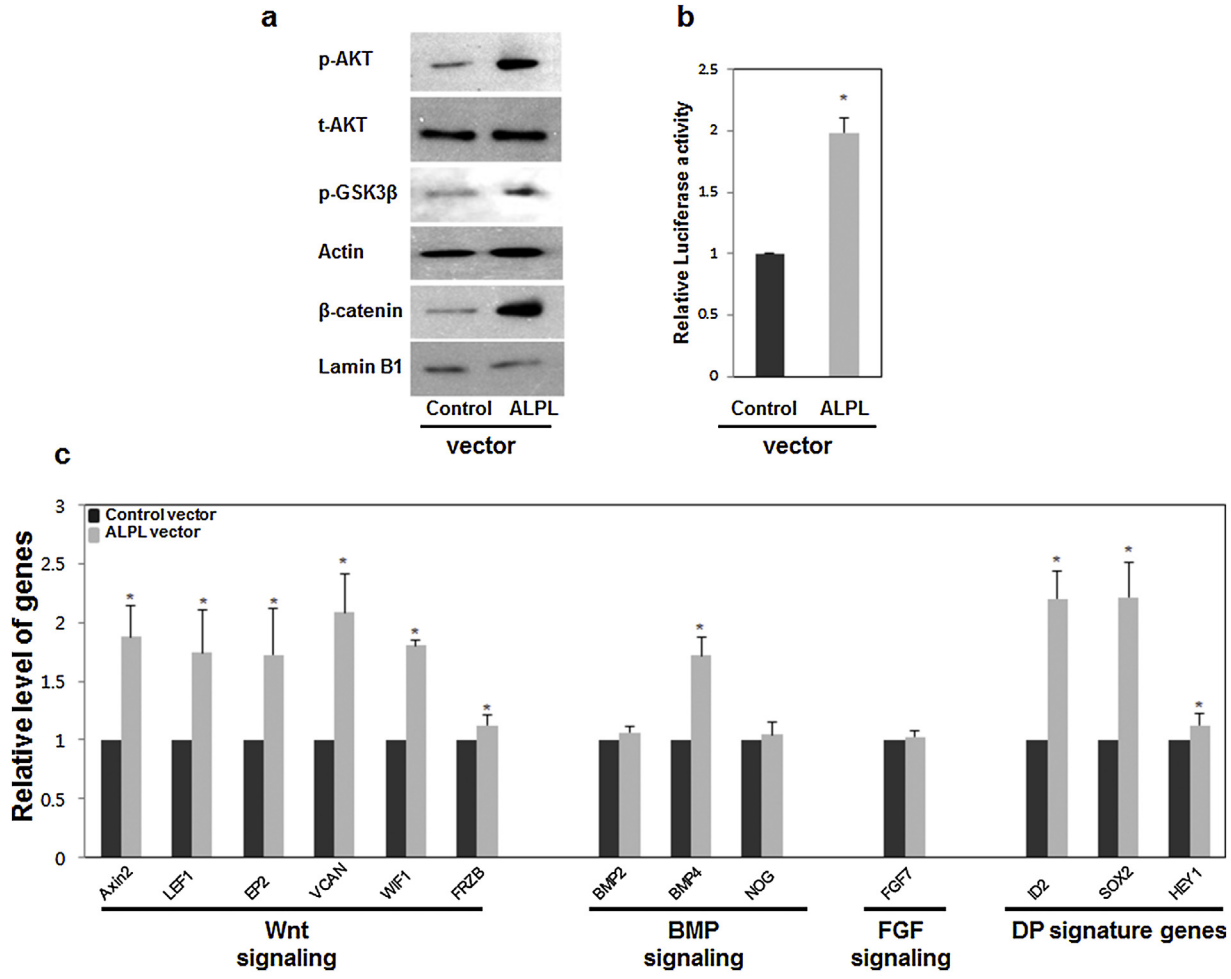

Fig. 2. Wnt/β-catenin signaling in DP spheres is regulated by ALPL. (a) Cultured human DP cells were transfected with the control vector or the *ALPL* expression vector for 48 h, and then seeded into a 96-well HydroCell plate and allowed to form spheres for 24 h. Total cell lysates of DP spheres were probed with p-AKT, AKT, or p-GSK3β antibodies, and nuclear fractions were probed with an anti-β catenin antibody. Actin and Lamin B1 levels were measured to assess the quantity and integrity of the protein samples of total cell lysates and nuclear fractions, respectively. (b) Cultured DP cells were transfected with the pTopflash plasmid and either the control vector or *ALPL* expression vector, and allowed to form spheres for 24 h prior to the luciferase assay. (c) Relative levels of representative DP signature genes, including those in the Wnt/β-catenin signaling pathway in control or ALPL vector-transfected DP spheres, were analyzed by real-time PCR. Data are expressed as the mean ± SD of three independent experiments performed in duplicate using three different DP lines ($^*P < 0.05$).

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.07.008.

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