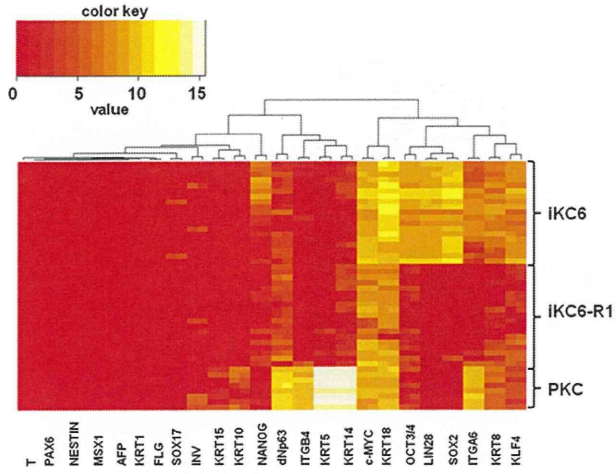


Figure 4. Single-cell gene expression analysis using the Fluidigm dynamic array. Single-cell gene expression profiling using Fluidigm dynamic arrays was performed. Heat maps depict the expression of genes differentially expressed between iKC6 (transgene residual) and iKC6-R1 (transgene-free) hiPSCs and primary human keratinocytes (control). Rows represent individual cells, and columns represent the evaluated genes. Genes highly expressed are shown in white, and genes with low expression are shown in red. Abbreviations: AFP, α-fetoprotein; dNp63, Δ N p63; iKC, induced keratinocyte; INV, involucrin; ITGA6, α6 integrin; ITGB4, β4 integrin; KRAT, keratin; PKC, primary human keratinocytes from neonatal male skin.

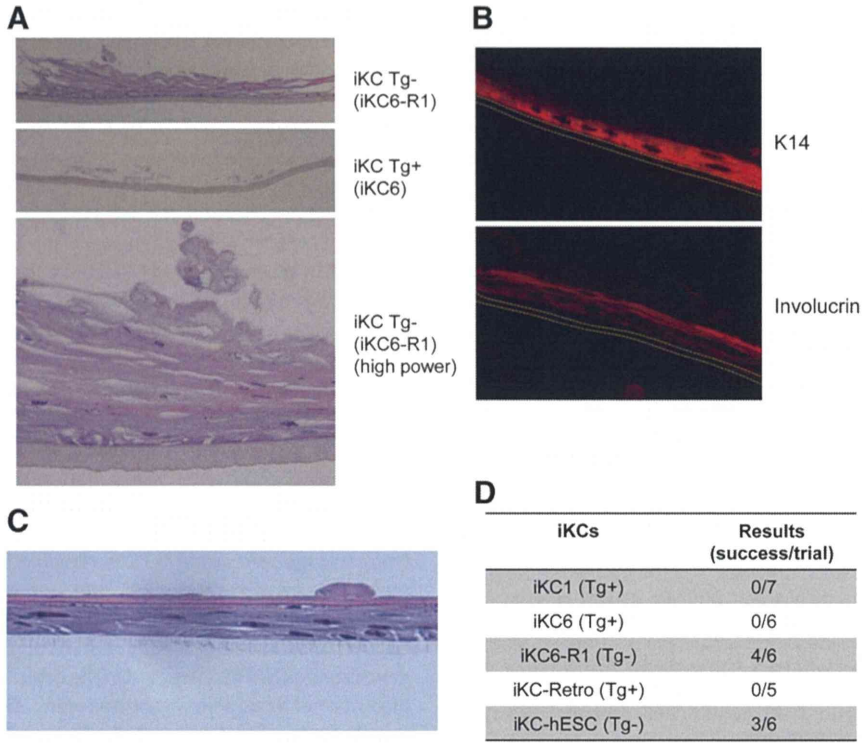
keratinocytes (CELLnTEC). According to the manufacturer's protocols, we performed the 3D culture experiments with both transgene-residual (iKC6) and -free (iKC6-R1) cell lines for approximately 21 days. As representatively shown in Figure 5A, iKC6-R1 cells successfully formed pluristratified epidermal structures in four of six experiments (67%), whereas no such structures were formed by iKC6 cells in any of the six experiments. In 3D culture, keratinocyte differentiation was evaluated by immunofluorescence staining of K14 and involucrin proteins (Fig. 5B). Lower layers of the pluristratified structure were positive for K14, whereas the upper layers were positive for involucrin, indicating in vitro reproduction of normal keratinocyte differentiation in vivo. A pluristratified structure was also formed by transgene-free hESCs in three of six experiments (50%) (Fig. 5C). In contrast, no such structures were formed by any of the transgene-residual hiPSC lines including iKC1 (seven experiments) and iKC-Retro (five experiments) (Fig. 5D). These results indicated that residual transgenes and their reactivation upon differentiation of keratinocytes from hiPSCs could critically influence not only the cellular phenotypes of such keratinocytes but also the functional properties of these cells for potential therapeutic use in regenerative medicine.

Discussion

In this study, we present a direct comparison of the effects of excising reprogramming transgenes from hiPSCs in terms of cellular and molecular phenotypes and their potential for differentiation into keratinocytes in vitro. Comparison between hiPSCs generated by different induction methods, e.g., retrovirus-based (transgene-residual) hiPSCs versus Sendai virus-based (transgene-free) hiPSCs, did not reveal any effects of residual transgenes in hiPSCs. However, our *piggyBac* transposon-based method allowed generation of an isogenic pair of hiPSC lines with or without retention of the reprogramming transgenes, leading to a precise evaluation of the effects of residual transgenes in hiPSCs and their derivatives.

The present study showed that, in the presence of the residual transgenes that had been silenced in a pluripotent state before differentiation induction, the iPSCs underwent transcriptional reactivation of the exogenous genes and showed less efficient differentiation into keratinocytes. In fact, we compared the phenotypes and function of transgene-residual and -free hiPSCs and their derivatives (iKCs). In the undifferentiated state, there appeared to be no characteristic differences between transgene-residual and -free hiPSCs. Next, we differentiated these established hiPSCs into epidermal keratinocytes using a modified method published elsewhere [19, 20]. In morphological, gene expression, and functional analyses, we found that transgene-residual hiPSCs did not fully differentiate into keratinocytes. Single-cell analysis using the Fluidigm dynamic array revealed that the cells derived from transgene-residual hiPSCs remained in an early developmental stage of keratinocyte differentiation, i.e., the K8/K18-positive stage, which may be related to residual transgene reactivation and subsequent activation of endogenous pluripotency genes such as NANOG (Fig. 4). On the other hand, cells that resembled normal human keratinocytes were effectively induced from transgene-free hiPSCs (Figs. 3–5). Single-cell analysis showed that our keratinocyte differentiation method successfully induced epidermal lineage cells, because cell types belonging to other lineages were not detected as shown in Figure 4. However, the expression of keratinocyte-specific genes such as K5, K14, and dNP63 was still weak even in cells differentiated from transgene-free hiPSCs compared with that in primary human keratinocytes, indicating that better methods need to be established for keratinocyte differentiation. Another explanation is that a minor population (~10%) of iKC cells that expressed an extremely high level of K14 as shown in Figure 3D was included in the bulk analysis but excluded from the single-cell analysis by random selection (Fig. 4).

In terms of the relationship between residual transgenes and iPSC function, we found conflicting results in the present study. Previous reports have suggested that impaired silencing of transgenes in hiPSCs results in poor differentiation [22, 23]. Once the transgenes were silenced in iPSCs, these cells appeared to show a normal differentiation ability. Major et al. [24] reported that there are no differences between neuronal cell differentiation from transgene-residual or -free hiPSCs (induced by a lentiviral vector with Cre-loxp-mediated transgene excision system), and in that study, transgenes were silenced in hiPSCs. On the other hand, Toivonen et al. [25] reported that the reactivation of transgene in retrovirally generated hiPSCs affected the differentiation ability of these cells. The major problems of these previous contradicted reports were that hiPSCs generated from different methods (i.e., retrovirus-based and Sendai virus-based hiPSCs) had been compared. However, with our present situation, we could compare the phenotypes of transgene-residual and -free hiPSCs of the same genetic background, and this led to a precise evaluation of the effects of residual transgenes in hiPSCs and their derivatives. Thus, in our study, although the transgenes appeared to be silenced in hiPSCs, reactivation of the transgenes was obvious upon keratinocyte differentiation, leading to poor keratinocyte differentiation.



iKCs	Results (success/trial)
iKC1 (Tg+)	0/7
iKC6 (Tg+)	0/6
iKC6-R1 (Tg-)	4/6
iKC-Retro (Tg+)	0/5
iKC-hESC (Tg-)	3/6

Figure 5. Functional analysis of iKCs from hiPSCs. **(A):** Representative cross-sectional views of three-dimensional (3D) culture of iKCs using a 3D epidermal culture system (CELLnTEC). iKC6 and iKC6-R1 keratinocytes were generated from KeI6 (Tg+) and KeI6-R1 (Tg−) hiPSCs, respectively. Hematoxylin and eosin staining. Magnification is ×40 (top and middle) and ×200 (bottom). **(B):** Immunofluorescence analysis of K14 and involucrin expression in the reconstituted epidermis shown in **(A)** (×100). **(C):** Representative cross-sectional view of 3D culture of iKCs from hESCs. Hematoxylin and eosin staining is shown at ×100 magnification. **(D):** Results of 3D culture-based functionality tests of iKCs derived from various hiPSC lines or a hESC line. Abbreviations: hESC, human embryonic stem cell; iKC, induced keratinocyte; iKC-hESC, iKCs from hESCs; Tg+, transgene-residual; Tg−, transgene-free.

From another point of view, Itoh et al. [20] also reported that they selected cells in which the transgenes were not reactivated when evaluating their keratinocytes induced from retrovirus-based hiPSCs. Based on these results, including our own, the differentiation abilities of transgene-residual hiPSCs may be impaired, and this phenomenon may be more easily detected depending on the induced cell type. Specifically, residual transgenes tended to be reactivated more easily upon keratinocyte differentiation.

To further investigate whether reactivation of residual transgenes in hiPSCs was related to the method of derivation, namely transposon system specific, we differentiated retrovirus-based hiPSCs that were already confirmed to be pluripotent [26] (transgene residual) and hESCs (no transgenes) into epidermal keratinocytes using the same protocols and performed characteristic analyses of these cells. Morphologically, as expected, the cells differentiated from retrovirus-based hiPSCs showed a spindle shape resembling that of the differentiated transposon-based transgene-residual hiPSCs. On the other hand, cells differentiated from hESCs showed a cobblestone appearance that resembled the morphology of transposon-based transgene-free hiPSCs. Moreover, we clearly observed transgene reactivation in cells differentiated from retrovirus-based hiPSCs. These results strongly suggest that residual transgenes in hiPSCs can affect the differentiation ability, at least when differentiating into keratinocytes, through the reactivation of residual transgenes.

Moreover, the piggyBac transposon-based system for cellular reprogramming allowed efficient removal of reprogramming transgenes without residual exogenous sequences or any footprint mutations in the hiPSC genome. Even for establishment of human disease models in vitro, proper quality and safety precautions may be required because of the use of hiPSCs with viral transgene integration. Reactivation of any integrated transgene is one of the reasons for the oncogenicity of iPSCs [5–8]. Furthermore, transgene integration itself causes insertional mutagenesis [9]. Therefore, several methods have been developed to generate iPSCs, other than retrovirus-based methods, including those using plasmids [27], recombinant proteins [28], episomal viral vectors [29], and mRNA [30, 31] for derivation of transgene integration-free iPSCs. Among these methods, although the Sendai viral vector is now widely used to generate transgene integration-free iPSCs, it can be quite difficult to show that there is no residual virus in the cells. Therefore, among the various methods, we selected the *piggyBac* transposon system to generate hiPSCs.

Conclusion

Our results have significant implications for the clinical use (or even laboratory use) of hiPSCs. Specifically, we confirmed that transgene-residual hiPSCs are not suitable for clinical use and that transgene integration-free hiPSCs are necessary. The timing of integrated transgene reactivation cannot be predicted, and thus, transgene-free hiPSCs are more appropriate not only for

clinical use and also laboratory use; otherwise the results may be affected. In addition, our *piggyBac* transposon system for the creation of hiPSCs may be a powerful approach, especially for clinical use.

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Author Contributions

K.I. and J.T.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; C.K. and K.H.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; K. Yusa: construction of reprogramming vectors, manuscript writing; Y.Y., K. Yamauchi, and H.S.: collection and/or assembly of data; H.Y. and I.K.: conception and design; M.T., N.K., H.O., Y.M., H.A., and A.U.: provision of retroviral-based hiPSCs.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Removal of Reprogramming Transgenes Improves the Tissue Reconstitution Potential of Keratinocytes Generated From Human Induced Pluripotent Stem Cells

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