Dact1 Regulates the Ability of 3T3-J2 Cells to Support Proliferation of Human Epidermal Keratinocytes

Daisuke Suzuki and **Makoto Senoo**[1]

Institute for Regenerative Medicine Department of Biomedical Sciences University of Pennsylvania School of Veterinary Medicine Philadelphia, Pennsylvania, USA

To the Editor

Efficient expansion of tissue-specific stem cells and progenitor cells is critical for therapeutic strategies in regenerative medicine. 3T3-J2 cells have been used clinically for expansion of human primary epidermal keratinocytes (HPEKs) (Green, 2008). While 3T3-J2 cells were originally derived from mouse embryonic fibroblasts (MEFs), they are vastly superior in their ability to support long-term keratinocyte proliferation (Supplementary Figure S1 online). Determining the mechanistic basis for this effect may identify the strategies to improve the efficacy of therapeutic expansion of epidermal keratinocytes.

As Wnt/β-catenin signaling stimulates keratinocyte proliferation (Lim *et al*., 2013), we first asked if increased Wnt expression by 3T3-J2 cells could contribute to long-term keratinocyte proliferation. However, gene expression analysis showed that 3T3-J2 cells and MEFs express Wnt ligands at similar levels (data not shown), suggesting that enhanced Wnt production by 3T3-J2 cells is unlikely to account for their superior ability to support keratinocyte proliferation. Unexpectedly, despite similar levels of Wnt receptors and inhibitors (data not shown), expression of *Axin2*, a well-characterized Wnt/β-catenin target was significantly lower in 3T3-J2 cells than in MEFs (Figure 1a), suggesting that Wnt/β-catenin signaling is reduced in 3T3-J2 cells. In support, we found that 3T3-J2 cells expressed a significantly higher level of the Wnt/β-catenin antagonist *Dact1* (Cheyette *et al*., 2002) than did MEFs (Figure 1b). As Dact1 can influence both Wnt-dependent and Wnt-independent signaling (Kivimäe *et al*., 2011), we next set out to determine if Dact1 expression influenced the ability of 3T3-J2 cells to support keratinocyte proliferation.

Notably, we found that silencing of *Dact1* in 3T3-J2 cells reduced keratinocyte proliferation with a corresponding reduction in epidermal clone size in clonogenic culture (Barrandon and Green, 1987) (Figure 1c-g and Supplementary Figure S2a-c online). As enforced Wnt/β-catenin signaling in 3T3-J2 cells by ectopically expressed Wnt/β-catenin ligands had similar effects on keratinocyte proliferation (Supplementary Figure S3 online), these results indicate

[1]To whom correspondence should be addressed. Phone: 215-746-2062 Fax: 215-573-5188 msenoo@vet.upenn.edu.

Conflict of Interest

The authors state no conflict of interest.

that Dact1 plays a critical role in the ability of 3T3-J2 cells to support keratinocyte proliferation, potentially by attenuating Wnt/β-catenin signaling in 3T3-J2 cells.

Although less proliferative, epidermal clones in *Dact1*-silenced 3T3-J2 cell culture expressed p63, a transcription factor essential for the proliferative potential of epidermal stem cells (Senoo *et al*., 2007), at a similar level as those in control culture (Figure 1h). Further, p63 phosphorylation, an early epidermal differentiation marker (Suzuki and Senoo, 2012), and expression of involucrin, a marker of terminal differentiation (Banks-Schlegel and Green, 1981), were similar in both cultures (Figure 1i-k). Moreover, keratinocytes from both cultures showed similar clonogenic potential in subsequent passages (Figure 1l and Supplementary Figure S2d online). These results indicate that loss of Dact1 in 3T3-J2 cells reduces proliferation of keratinocytes without promoting their differentiation.

As transforming growth factor-β (Tgfβ) also inhibits proliferation of keratinocytes without influencing their differentiation (Shipley *et al*., 1986), we next determined if the Dact1-dependent regulation of keratinocyte proliferation by 3T3-J2 cells involved Tgfβ. As HPEKs express Wnt/β-catenin ligands (Lim *et al*., 2013), we first asked if *Dact1*-silencing influenced Tgfβ expression in response to exogenous Wnt stimulation. Notably, silencing of *Dact1* in 3T3-J2 cells enhanced expression of Tgfβ2, but not Tgfβ1 or Tgfβ3, in response to Wnt3A stimulation compared to control cells at both the mRNA and protein levels (Figure 2a and b). However, the basal Tgfβ2 expression was unchanged in *Dact1*-silenced 3T3-J2 cells (Figure 2a and b), suggesting that increased Tgfβ2 expression by 3T3-J2 cells required additional Wnt/β-catenin stimuli provided by HPEKs. In support, treatment of 3T3-J2 cells with HPEK-conditioned media stimulated *Tgβ2* expression while this effect was significantly suppressed when HPEK-conditioned media were prepared in the presence of LGK974, an inhibitor of Porcupine essential for Wnt secretion (Liu *et al*., 2013) (Figure S4). These results suggest that in our co-culture system, Dact1 counteracts Tgfβ2 upregulation by 3T3-J2 cells in response to HPEK-derived Wnt ligands, thereby preventing Tgfβ2-mediated inhibition of keratinocyte proliferation.

Among known growth-promoting factors, insulin-like growth factors (Igfs) have been implicated in the ability of 3T3-J2 cells to promote keratinocyte proliferation (Barreca *et al*., 1992). Consistent with these findings, silencing of *Dact1* in 3T3-J2 cells reduced Igf2 at both the mRNA and protein levels while Igf1 was undetectable (Figure 2c and d), suggesting that Dact1 plays an important role in the transcriptional control of Igf2 in 3T3-J2 cells. Moreover, Tgfβ signaling can suppress Igf expression in human mesenchymal stem cells and other murine fibroblasts (Ochiai *et al*., 2012). Notably, we found that Tgfβ2 treatment further reduced *Igf2* expression in *Dact1*-silenced 3T3-J2 cells, but less so in control 3T3-J2 cells (Figure 2c). These results suggest that Dact1 also attenuates Tgfβ signaling, thereby maintaining the relatively high levels of Igf2 critical for 3T3-J2 cell-mediated keratinocyte proliferation.

In further support of Dact1-mediated attenuation of Tgfβ signaling, we found that Smad2/3 phosphorylation, an indicator of activated Tgfβ signaling (Kamato *et al*., 2013), was higher in *Dact1*-silenced 3T3-J2 cells than in control cells in response to Tgfβ signaling (Figure 2e and f). Further, treatment with RepSox, a Tgfβ receptor type I kinase inhibitor (Ichida *et al*.,

2009), restored *Igf2* levels in *Dact1*-silenced 3T3-J2 cells in a dose-dependent manner (Figure 2g). These results indicate that Dact1 counteracts Tgfβ signaling, which otherwise reduces Igf2 levels in 3T3-J2 cells.

Although 3T3-J2 cells have been utilized extensively in the last ~50 years in both stem cell biology and regenerative medicine, their extraordinary capacity to support keratinocyte proliferation is not yet understood. By dissecting signaling pathways, we have revealed the existence of a uniquely configured mechanism in 3T3-J2 cells that optimizes support for keratinocyte proliferation. Our present study shows that Dact1 plays a critical role in this process, likely through attenuation of both Wnt-induced Tgfβ2 expression and Tgfβ-mediated downregulation of Igf2 expression in 3T3-J2 cells (Figure 2h). Although the involvement of other signaling pathways and molecules need to be fully investigated, our current findings hold important implications for dissecting gene programs controlling keratinocyte proliferation and improving the efficacy of therapeutic expansion of epidermal keratinocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations

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Dact1	dapper antagonist of β-catenin 1
HPEK	human primary epidermal keratinocyte
Igf	insulin-like growth factor
MEF	mouse embryonic fibroblast
Tgfβ	transforming growth factor-β

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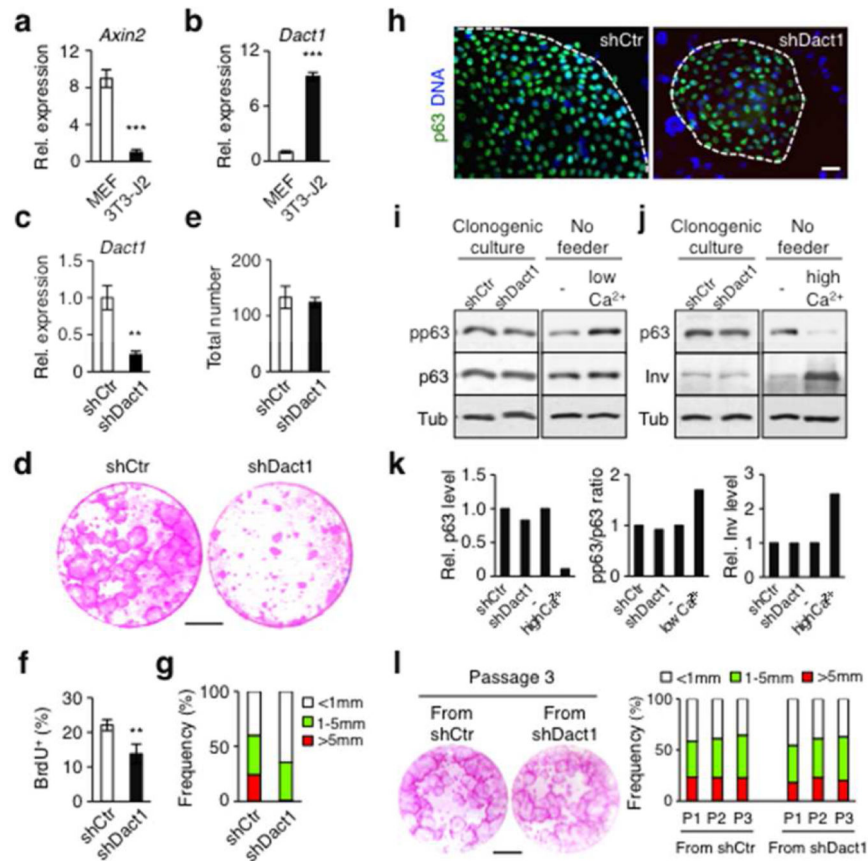
Figure 1. Suppression of *Dact1* in 3T3-J2 cells reduces clonogenic expansion of HPEKs
(**a**) Relative expression of *Axin2*. (**b**) Expression of *Dact1* in 3T3-J2 cells. (**c**) Suppression of
Dact1 by shRNA (shDact1) in 3T3-J2 cells. *shCtr*, control shRNA. (**d**) Rhodamine staining
of epidermal clones grown for 14 days. Bar=10 mm. (**e**) Total number of epidermal clones at
day 14. (**f**) Proliferation of keratinocytes in clonogenic culture at day 7. (**g**) Size distribution
of epidermal clones at day 14. (**h**) Expression of p63 in representative epidermal clones.
Bar=20 μm. (**i** and **j**) Western blot of keratinocytes from clonogenic cultures. HPEKs
stimulated with low (**i**: 0.3 mM) or high (**j**: 1.3 mM) calcium, as positive controls. (**k**)
Quantification of **i** and **j**. (**l**) Serial culture of equal plated numbers of keratinocytes, grown
for 14 days with γ-irradiated fresh 3T3-J2 cells. *Left*: rhodamine staining at passage (P) 3.
Right: size distribution of epidermal clones over multiple passages. Bar=10 mm. Data shown
are mean±SEM (n=3). **P<0.01; ***P<0.001.
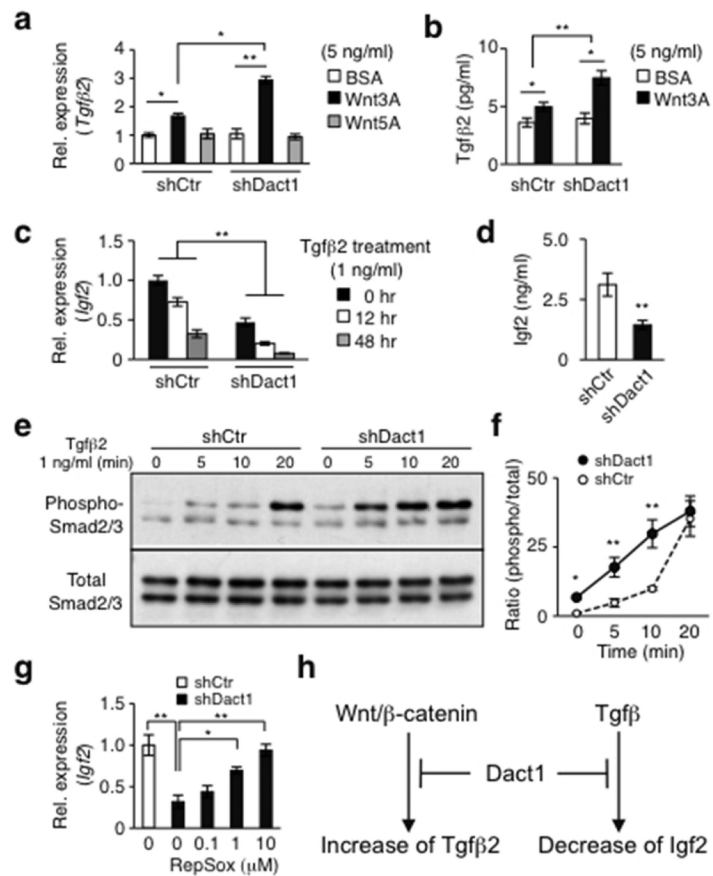
Figure 2. Dact1 attenuates Wnt and Tgfβ signaling in 3T3-J2 cells
(**a** and **b**) Enhanced Tgfβ2 mRNA (**a**) and protein (**b**) levels in *Dact1*-silenced 3T3-J2 cells
at day 4 by Wnt3A. (**c**) Reduced *Igf2* expression by loss of Dact1 and further suppression by
Tgfβ2 treatment in *Dact1*-silenced 3T3-J2 cells. (**d**) Decreased basal Igf2 protein levels in
Dact1-silenced 3T3-J2 cells. (**e** and **f**) Enhanced Smad2/3 phosphorylation in *Dact1*-silenced
3T3-J2 cells. (**g**) Recovery of *Igf2* expression by RepSox in *Dact1*-silenced 3T3-J2 cells. (**h**)
Model for the role of Dact1. Data shown are mean±SEM (n=3). *$P<0.05$; **$P<0.01$.