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# The δ-Opioid Receptor Affects Epidermal Homeostasis via ERK-Dependent Inhibition of Transcription Factor POU2F3

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Neuropeptides and their receptors are present in human skin, and their importance for cutaneous homeostasis and during wound healing is increasingly appreciated. However, there is currently a lack of understanding of the molecular mechanisms by which their signaling modulates keratinocyte function. Here, we show that δ-opioid receptor (DOPr) activation inhibits proliferation of human keratinocytes, resulting in decreased epidermal thickness in an organotypic skin model. DOPr signaling markedly delayed induction of keratin intermediate filament (KRT10) during *in vitro* differentiation and abolished its induction in the organotypic skin model. This was accompanied by deregulation of involucrin (IVL), loricrin, and filaggrin. Analysis of the transcription factor POU2F3, which is involved in regulation of KRT10, IVL, and profilaggrin expression, revealed a DOPr-mediated extracellular signal-regulated kinase (ERK)-dependent downregulation of this factor. We propose that DOPr signaling specifically activates the ERK 1/2 mitogen-activated protein kinase pathway to regulate keratinocyte functions. Complementing our earlier studies in DOPr-deficient mice, these data suggest that DOPr activation in human keratinocytes profoundly influences epidermal morphogenesis and homeostasis.

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## INTRODUCTION

The epidermis is a stratified epithelium constantly undergoing self-renewal, which is temporally and spatially coordinated by the balanced expression of genes regulating proliferation and differentiation of keratinocytes, the main cell type present (Blanpain and Fuchs, 2009). The transition of basal keratinocytes toward the spinous layer is accompanied by repression of the synthesis of intermediate filament proteins keratin 5 (KRT5) and KRT14 (Fuchs and Green, 1980) and the upregulation of early differentiation markers KRT1 and KRT10. Differentiation toward the granular layer involves upregulation of cornified envelope precursor proteins such as involucrin (IVL) and loricrin (LOR), as well as filaggrin (FLG).

This sequence of epidermal gene regulation required for appropriate differentiation of keratinocytes is regulated by several transcription factors, including POU domain, class 2, transcription factor 3 (POU2F3, also known as Skn-1, Epoc-1, and Oct-11). POU2F3 belongs to a family of POU domain transcription factors, which are preferentially expressed in specific epidermal layers and are involved in regulation of multiple keratinocyte differentiation genes. POU2F3 protein seems to be expressed throughout all epidermal layers with highest expression in the suprabasal layers (Andersen *et al.*, 1993; Goldsborough *et al.*, 1993; Yukawa *et al.*, 1993; Faus *et al.*, 1994; Andersen *et al.*, 1997), and mice lacking POU2F3 express normal levels of KRT1 and KRT10 but show marked abnormalities in *KRT14* gene expression during wound healing. POU2F3 gene expression is spatially regulated at the wound front, corresponding to altered *KRT1*, *LOR*, *FLG*, *KRT14*, and *SPR1* gene expression, which suggests a role for POU2F3 in facilitating reepithelialization at the wound front (Andersen *et al.*, 1997).

In view of the constant environmental assaults that the skin must endure, such a delicate balance of gene expression might easily become derailed in the absence of robust stabilizing mechanisms. Recently, attention has focused on the local skin neuroendocrine system as a potential player in regulating epidermal differentiation. Expression of several neurohormones, neurotransmitters, and neuropeptides, including β-endorphin and enkephalins, has been shown in human skin (Slominski *et al.*, 2000; Slominski and Wortsman, 2000; Bigliardi-Qi *et al.*, 2000; Kauser *et al.*, 2003; Bigliardi-Qi

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*Abbreviations: DOPr, δ-opioid receptor; ERK, extracellular signal-regulated kinase; FLG, filaggrin; GFP, green fluorescent protein; IVL, involucrin; KRT, keratin intermediate filament; LOR, loricrin; Met, methionine[5]; POU2F3, POU domain, class 2, transcription factor 3*

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*et al.*, 2004; Slominski *et al.*, 2011, 2012, 2013). We are particularly interested in the role of the receptor for methionine[5] (Met-) and leucine-enkephalin, the δ-opioid receptor (DOPr), in skin healing and homeostasis (Bigliardi *et al.*, 2009). Following specific activation at the cell surface, the DOPr transmits the signal across the membrane via an associated G-protein, leading to intracellular signaling cascade activation. The DOPr can regulate extracellular signal-regulated kinase (ERK) 1/2 activity (Burt *et al.*, 1996; Fukuda *et al.*, 1996; Belcheva *et al.*, 1998; Xu *et al.*, 2010), which has been linked to cell differentiation and proliferation (Eckert *et al.*, 2002; Shaul and Seger, 2007; Gazel *et al.*, 2008). Expression of the DOPr in murine and cultured human skin has been documented, and mice lacking the DOPr exhibit aberrant epidermal phenotypes and delayed wound healing (Bigliardi-Qi *et al.*, 2006). Although the mechanisms underlying these phenotypes remain unclear, the importance of the DOPr in these processes is only now beginning to be appreciated: DOPr-deficient mice exhibit markedly increased expression of KRT10, alongside an atrophic epidermis, alluding to a role for DOPr during stratification and skin homeostasis (Bigliardi-Qi *et al.*, 2006, 2009).

In this study, we sought to identify the molecular consequences of DOPr activation in keratinocytes and to understand how this influences skin differentiation and homeostasis.

## RESULTS

### DOPr is expressed in suprabasal and granular layers of human epidermis

We have previously documented the expression of DOPr mRNA in cultured human keratinocytes (Bigliardi-Qi *et al.*, 2006, 2009), and have now gone on to confirm this expression *in vivo* by *in situ* hybridization on human corporal skin sections. Positive hybridization signals were detected in the stratum granulosum and, to a lesser extent, in the stratum spinosum. However, it was apparent that not all keratinocytes express the same amount of DOPr, reflected in the heterogeneous staining pattern (Figure 1a).

Further, to reliably identify the localization of the receptor, a lentiviral overexpression system was used to introduce a DOPr–green fluorescent protein (GFP) fusion protein into N/TERT-1 keratinocytes. In low $Ca^{2+}$ (0.09 mM) medium, DOPr in cultured keratinocytes was almost completely localized in intracellular compartments, with little expression at the cell surface (Figure 1b—column 1). Upon shifting DOPr-overexpressing keratinocytes to higher $Ca^{2+}$ concentrations (1.2 mM), the majority of DOPr translocated to the cell surface, and a smaller fraction was detected in intracellular compartments (Figure 1b—column 5). Within 1 hour of addition of $Ca^{2+}$, the opioid receptor was found on the membrane, despite the cells having not yet fully established desmosomal junctions, marked by desmoplakin labeling at areas of cell–cell contact (Figure 1b—column 3). Eight hours after addition of high $Ca^{2+}$, both desmosomal junction formation and DOPr membrane localization had stabilized (Figure 1b—column 4).

### Overexpression and activation of the DOPr results in reduced proliferation of keratinocytes

DOPr overexpression markedly changed the phenotype of N/TERT-1 keratinocyte cultures. Colonies of DOPr-overexpressing cells were more spread out than control cell colonies and appeared to have reduced cell proliferation rates. Although control cells entered an exponential growth phase, before plateauing after about 6 days in culture, DOPr-overexpressing cells showed markedly reduced proliferation (Figure 2a). The addition of the DOPr ligand SNC80 significantly and specifically reduced the level of confluence of DOPr-overexpressing cell cultures (Figure 2b).

### DOPr activation alters keratinocyte differentiation

We further went on to analyze the changes in expression of KRT10 and KRT1 in an *in vitro* model of keratinocyte differentiation using N/TERT-1 keratinocytes (Dickson *et al.*, 2000). Cells were grown to confluence before differentiation was induced by growth factor withdrawal for up to 10 days in the presence of the DOPr peptide agonist Met-enkephalin. Quantitative real-time PCR analysis of mRNA levels demonstrated that continued differentiation was associated with 56- (KRT10) and 114-fold (KRT1) upregulation of these transcripts in control cells, whereas DOPr-overexpressing cells exhibited significantly lower inductions; just 4- (KRT10) and 13-fold (KRT1) increases were observed (Figures 3a and b). With advancing differentiation, the mRNA levels of the cornified envelope precursor proteins, IVL, LOR, and FLG, increased markedly in control cells, but in DOPr-overexpressing cells only at day 10 was a comparable increase in expression of these genes detected (Figures 3c–e).

The delay in KRT1 and KRT10 induction during early differentiation under the influence of DOPr signaling was confirmed at the protein level. The increase in KRT1 and KRT10 observed in control cells at day 4 and 7 of differentiation was not detected in DOPr-activated cells in the presence of the endogenous agonist Met-enkephalin (Figure 3f). When DOPr-overexpressing cells were differentiated without addition of an agonist, a minor reduction in KRT10 protein expression was also detected (Figure 3f, lanes 11–14). Similar results were obtained using the exogenous DOPr agonist SNC80 (Figure 3g, lanes 5–8), further illustrating the importance of the DOPr pathway irrespective of the ligand used to stimulate signaling.

### The transcription factor POU2F3 is involved in DOPr-mediated regulation of differentiation

POU2F3 is one transcription factor involved in the transition from basal to suprabasal keratinocyte phenotype (Lena *et al.*, 2010) and in the regulation of KRT10 and IVL expression (Andersen *et al.*, 1993; Welter *et al.*, 1996; Andersen *et al.*, 1997). Our observations in HaCaT cells (Supplementary Figures S3a and S3c online), $DOPr^{-/-}$ mice, and activated DOPr-overexpressing N/TERT-1 keratinocytes (Figure 4a) confirm these published reports. After 1 day of differentiation, DOPr activation by Met-enkephalin was associated with a 1.3-fold downregulation of POU2F3 mRNA, whereas control cells induced POU2F3 transcription 1.5-fold. The relative