OSMRβ mutants enhance basal keratinocyte differentiation via inactivation of the STAT5/KLF7 axis in PLCA patients

Letter

Dear Editor,

Primary localized cutaneous amyloidosis (PLCA) is a skin-limited disorder characterized by deposition of amyloid material in the superficial dermis. According to clinical characteristics, PLCA is divided into lichen, macular, and nodular amyloidosis. PLCA is found worldwide but has a higher incidence in South America and Southeast Asia, such as in Brazil and China (Chang et al., 2014; Tey et al., 2016). The etiology of PLCA is complicated, involving environmental factors, the immune state, and genetic factors (Tanaka et al., 2009; Katayama et al., 2019). A genome-wide scan revealed that mutations in several genes are involved in the development of PLCA, including oncostatin M receptor (OSMR) (Arita et al., 2008), interleukin 31 receptor A (IL-31RA) (Shiao et al., 2013), and glycoprotein Nmb (GPNMB) (Yang et al., 2018).

Recently, we demonstrated that the c.1538G>A (p.G513D) and c.2081C>T (p.P694L) mutations of OSMR were the most frequent mutations in a Chinese PLCA population (Lu et al., 2019). It has been reported that OSM maintains hair follicle stem cell and muscle stem cell quiescence by binding to heterodimeric receptors comprising gp130 and OSMRβ (Sampath et al., 2018; Wang et al., 2019). Additionally, OSM signaling plays crucial roles in the regulation of cardiomyocyte differentiation and cellular plasticity (Kubin et al., 2011). Whether OSMRβ-mediated cell differentiation plays a role in PLCA remains unexplored.

To answer this question, we compared the RNA expression profiles between PLCA patients and healthy controls. Interestingly, Gene Ontology (GO) analysis of the dysregulated genes revealed that most of them were associated with keratinocyte differentiation processes (Fig. 1A). Consistent with the above findings, in PLCA patients with OSMR mutations, epidermal keratinocyte differentiation was enhanced, with increased expression of FLG and LOR, compared to that in healthy controls (Fig. 1B). Furthermore, immunofluorescence analysis suggests that the expression of Ki67, an indicator of cell proliferation, was also enhanced in the epidermis of PLCA patients with OSMR mutations (Fig. 1C and 1D).

To further determine the biological functions of OSMRβ protein in the skin, Osmr−/− C57BL/6 mice were produced using the CRISPR/Cas9 system (Figs. 1E, 1F and S1A). Unfortunately, no PLCA-like phenotype was observed in these mice under physiological or pathological conditions (including UVA exposure and an itch challenge; data not shown). Hair follicle cycle changes were observed between WT and Osmr−/− mice at post-natal day 30 (P30) using hematoxylin and eosin staining (Fig. S1B), which is consistent with previous reports (Wang et al., 2019). More importantly, the tail epidermal thickness was significantly
increased in Osmr−/− mice compared to WT mice (Fig. 1G and 1H). RNA-seq analysis indicated a 2-fold change in the expression levels of 2,328 genes in the skin of Osmr−/− mice compared to their WT littermates. GO analysis showed that the genes related to certain functions, such as keratinocyte differentiation and skin development, were dysregulated, which suggests that epidermal keratinocyte differentiation was enhanced in the skin of Osmr−/− mice (Fig. S1C). Of the differentially expressed genes between the two groups, 39 genes are known to be related to epidermal keratinocyte differentiation (Fig. 1I). To validate our findings, we used qRT-PCR to examine terminal differentiation marker expression levels of their WT littermates. This analysis confirmed increasing expression in freshly isolated skin from Osmr−/− mice and their WT littermates. These results suggest that Osmr knockout enhances basal keratinocyte differentiation and proliferation in mice.

OSMRβ is a component of both the OSM type II receptor and the IL-31 receptor, so we sought to investigate which of these cytokines was involved in the regulation of human keratinocyte differentiation. HaCaT and primary keratinocytes were cultured and stimulated with indicated concentration of OSM or IL-31, and the expression levels of epidermal keratinocyte differentiation-related genes were significantly decreased in the OSM-treated HaCaT cells or primary keratinocytes (Figs. 2A, 2B and S2A–D). Further, we validated our results using 3D skin models. Consistently, qRT-PCR and immunofluorescence analysis demonstrated the decreased expression of epidermal keratinocyte differentiation markers in OSM-treated 3D skins (Figs. 2C and S2E).

To further confirm that OSM inhibits keratinocyte differentiation through heterodimeric receptors comprising gp130 and OSMRβ, the CRISPR/Cas9 system was employed to produce OSMR-knockout HaCaT cells (Fig. S2F–H).
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A. Protein & Cell

B. Healthy control vs. PLCA with OSMR mutation

C. Krt14, KI67, Integrin α6, DAPI, Merge

D. % KI67+ (Krt17+ basal keratinocytes)

E. 5′ gRNA1 4 5 6 18

F. Mouse Osmr Intron 3

G. Osmr+−

H. Epidermal thickness (μm)
Figure 1. continued.
results indicated that knockout of OSMR can rescue OSM-induced inhibition of keratinocyte differentiation (Fig. 2D). Next, we aimed to identify the molecular mechanisms underlying OSM-induced inhibition of keratinocyte differentiation. Western blot showed that HaCaT cells exhibited activation of STAT3, STAT5, ERK1/2, and AKT signaling after OSM stimulation (Fig. S2I). However, only the STAT3 and ERK1/2 pathways were activated in IL-31-treated HaCaT cells (Fig. S2I). We further demonstrated that OSMR knockout can block OSM-induced phosphorylation of STAT3, STAT5, ERK1/2, and AKT in HaCaT cells (Fig. 2E).

In an attempt to delineate the downstream signaling pathways, HaCaT cells were pretreated with inhibitors before being stimulated with OSM. STAT5 inhibitor could almost completely rescue the decreased mRNA expression of keratinocyte differentiation-related genes (Fig. S2J). STAT3 inhibitor had a partial rescue effect (Fig. S2K). And ERK1/2 (Fig. S2L) and AKT (Fig. S2M) inhibitors had no effect at all. Similar results were observed when the protein expression levels of these differentiation genes were checked (Figs. 2F and S2N). These findings strongly suggest that OSM/OSMRβ signaling, likely via JAK/STAT5, is involved in the regulation of keratinocyte differentiation.

Next, we tried to identify critical factors downstream STAT5 to regulate keratinocyte differentiation. An increased abundance of KLF7 (Fig. 2G), a transcription factor involved in the regulation of somatic stem cell quiescence (Wang et al., 2016), was observed in OSM-treated HaCaT cells. In contrast, decreased Klf7 expression was found in OsMr−/− mice compared to WT mice (Fig. S3A). OSM-induced upregulation of this gene in mRNA and protein in HaCaT cells was further identified using qRT-PCR and Western blot, respectively (Figs. 2H and S3B).

Further, we analyzed two available STAT5 ChIP-seq data sets involving human B lymphocytes (Gertz et al., 2013) and mice natural killer cells (Villarino et al., 2017). The results indicated that human and mice STAT5-binding sites at the KLF7 intragenic locus are highly conserved (Fig. S3C and S3D). Furthermore, ChIP-qPCR confirmed that STAT5 binds to the KLF7 gene locus in human keratinocytes upon OSM stimulation (Fig. 2I). In addition, we cloned approximately 2 kb of the upstream region of the KLF7 gene transcriptional start site (defined as KLF7 promoter in our study) into a pGL4 luciferase reporter vector, and we then tested whether the luciferase reporter activity is regulated by STAT5 in HEK293T cells. As shown in Fig. 2J, the upregulation of luciferase activity in OSM-stimulated HEK293T cells was inhibited, in a dose-dependent manner, by pretreatment with a STAT5 inhibitor, but not by pretreatment with STAT3, ERK1/2, or AKT inhibitors. Three potential STAT5-binding sites (ChIP-seq peaks) were found within the KLF7 promoter region, combinational deletion experiments demonstrated that all the three sites contributed to KLF7 expression (Fig. S3E). These above data indicate that KLF7 is a direct target gene of STAT5.

To investigate the function of KLF7 in keratinocyte differentiation, KLF7-overexpressing lentivirus was packaged and transduced into HaCaT cells. As expected, qRT-PCR and Western blot revealed that the expression levels of KRT1, KRT10, FLG, and LOR were decreased in KLF7-overexpressing HaCaT cells (Fig. 2G). Two independent siRNAs resulted in efficient knockdown of KLF7 (Fig. S3H) and in a reduction of OSM-induced keratinocyte differentiation (Fig. S3I). To further confirm our results, CRISPR/Cas9 technology was employed to generate KLF7-knockout HaCaT cell lines (Fig. 2K). Loss of KLF7 also resulted in the inhibition of OSM-induced keratinocyte differentiation (Fig. 2L). These findings support our hypothesis that OSM inhibits keratinocyte differentiation through activation of the STAT5/KLF7 signaling pathway.

Several studies have demonstrated that missense mutations of OSMR are involved in the development of PLCA (Arita et al., 2008; Wali et al., 2015). However, the changes in the biological function of the OSMR protein caused by the mutations still need to be identified. The OSMR-knockout HaCaT cell line was infected with Lv-OSMR-pG513D-P2A-GFP, Lv-OSMR-pP694L-P2A-GFP, or the Lv-OSMR-WT-P2A-GFP virus as control (Fig. S3J). We assessed whether the p.G513D and p.P694L variants resulted in mislocalization of OSMRβ using immunofluorescence analysis. The
results showed that the cellular localization of WT-OSMRβ and the two variants (with the p.G513D and p.P694L mutations) were similar, indicating that neither variant impacted the overall cellular localization (Fig. S3K). We next checked the downstream signaling pathways activated by the two variants. Western blot showed that p.P694L mutant failed to activate STAT5 and STAT3, and that p.G513D mutant failed to activate STAT5 (Fig. 2M). No dominant negative effect was observed, as OSM can activate either STAT5 or STAT3 phosphorylation in both WT/p.G513D and WT/p.P694L co-infected HaCaT cells (Fig. S3L). More importantly, qRT-PCR analysis further confirmed that the OSMR mutations resulted in the inhibition of OSM-induced keratinocyte differentiation (Fig. 2N).

In summary, we identified OSM as a negative regulator of epidermal keratinocyte differentiation that acts via STAT5/ KLF7 signaling in vivo and in vitro. Dysregulation of the OSM/OSMRβ/STAT5/KLF7 axis by OSMR mutation could lead to PLCA (Fig. S4). Therefore, this study discovered the potential underlying cellular and molecular mechanisms how OSMR mutations caused PLCA, and the discovered STAT5/ KLF7 molecule axis could be a potential target for PLCA treatment in the future.

FOOTNOTES

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Jun Liu, Junchen Chen, Yadan Zhong, Xiaoling Yu, Jianqi Feng, Xin Zhang, Shufeng Ma, Chao Yang, Bin Yang, and Zhili Rong declare that they have no conflict of interest. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (the Dermatology Hospital of Southern Medical University) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study. All institutional and national guidelines for the care and use of laboratory animals were followed.
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**Figure 1:**

**A** Western blot analysis showing the expression of OSMRβ mutants in IL-31 and KRT10

**B** Heatmap representing the expression levels of various genes in KC and KC + OSM

**C** Immunofluorescence images of 3D skin, 3D skin + OSM, and 3D skin + IL-31

**D** Bar graph showing relative mRNA levels of OSMR KO and OSMR KO + OSM

**E** Western blot analysis of HaCaT and OSMR KO

**F** Western blot analysis of STAT5-Stat5

**G** Volcano plot showing the expression of KLF7

**H** Western blot analysis of OSM and IL-31

**I** Bar graph showing signal relative to input of KLF7 intragenic locus

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Figure 2. continued.
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