Stem Cell Research 29 (2018) 15–23

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Epidermal YAP activity drives canonical WNT16/β-catenin signaling to promote keratinocyte proliferation *in vitro* and in the murine skin

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ARTICLE INFO

Article history:
Received 24 October 2017
Received in revised form 7 February 2018
Accepted 7 March 2018
Available online 10 March 2018

Keywords:
YAP
WNT signaling
Epidermis
Regeneration

ABSTRACT

The skin constantly self-renews throughout adult life. Wnt/β-catenin signaling plays a key role in promoting keratinocyte proliferation in the hair follicles and in the interfollicular epidermis. A recent report demonstrated that epidermal YAP activity drives β-catenin activation to promote keratinocyte proliferation in the murine skin. However, it remains unclear whether this is caused by paracrine activation of canonical Wnt signaling or through other YAP/β-catenin regulatory interactions. In the present study, we found that XAV939-inhibition of canonical WNT signaling in skin of YAP2-5SA-ΔC mice resulted in diminished β-catenin activation, reduced keratinocyte proliferation, and a mitigation of the hyperplastic abnormalities in the interfollicular epidermis, signifying a canonical WNT ligand-dependent mechanism. Our subsequent analyses determined that WNT16 is produced in response to YAP activity in keratinocytes both *in vitro* and *in vivo*, and that WNT16 drives HaCaT keratinocyte proliferation *via* canonical WNT16/β-catenin signaling. We conclude that under normal physiological conditions WNT16 is the paracrine WNT ligand secreted in response to epidermal YAP activity that promotes cell proliferation in the interfollicular epidermis. This study delineates a fundamental YAP-driven mechanism that controls normal skin regeneration, and that may be perturbed in human regenerative disease displaying increased YAP and WNT signaling activity.

1. Introduction

The skin is a unique organ characterized by its continuous regeneration. In order to maintain normal skin homeostasis, epidermal stem/progenitor cell proliferation must be tightly regulated, and any variation of this well-balanced process may result in the development of skin disease. However, the molecular mechanisms involved in epidermal stem/progenitor cell proliferation remain poorly understood.

The Hippo kinase pathway controls organ development, tissue regeneration and stem/progenitor cell self-renewal (Camargo et al., 2007; Dong et al., 2007; Lian et al., 2010). This kinase cascade is classically known to phosphorylate the downstream effectors Yes-Associated Protein (YAP) and the transcriptional co-activator with PDZ-binding motif (TAZ), resulting in their inactivation through cytosolic retention. Unphosphorylated YAP/TAZ translocate to the nucleus and bind the Transcriptional Enhancer Associate Domain (TEAD) transcription factors to activate gene expression and cell proliferation. Recently, mechanical factors and the tissue microenvironment were shown to play a major overarching role over the Hippo kinase pathway in controlling YAP/TAZ activity, which since then are known to act as mechanosensors in control of tissue homeostasis (Aragona et al., 2013; Dupont et al., 2011; Piccolo et al., 2014; Yu et al., 2015).

The canonical WNT pathway also has a crucial role during development, cell fate determination and stem/progenitor self-renewal in many tissues (Clevers, 2006). WNT signaling is activated by binding of WNT ligands to the Frizzled transmembrane receptors. This results in the dissociation of β-catenin from the cytosolic destruction complex, and its nuclear translocation and binding to TCF/LEF family transcription factors to activate the expression of target genes and epidermal stem/progenitor cell proliferation (Clevers et al., 2014; Huelsken et al., 2001; Lo Celso et al., 2004). Non-canonical WNT pathways such as the planar cell polarity and WNT/calcium pathways act independently of the cytosolic destruction complex and β-catenin transcriptional activity, and mainly control cellular polarity and motility (Katoh, 2005; Kohn and Moon, 2005).

Canonical WNT/β-Catenin signaling has well-established roles in the activation of keratinocyte proliferation during hair follicle cycling (Huelsken et al., 2001). Furthermore, recent reports have resolved a longstanding contentious issue in the field, and unequivocally established that paracrine Wnt signaling and β-catenin transcriptional activity are required to promote basal keratinocyte proliferation in the interfollicular epidermis (Choi et al., 2013; Lim et al., 2013), and not

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https://doi.org/10.1016/j.scr.2018.03.005

keratinocyte differentiation, as initially thought (Huelsken et al., 2001). However, the WNT ligands that are responsible for driving β-catenin transcriptional activity in the interfollicular epidermis remain unknown.

The Hippo/YAP pathway interacts with many signaling pathways to control the homeostasis of tissues, including in the skin (Akladios et al., 2017a,b; Piccolo et al., 2014). Cross-talk between the Hippo/YAP and WNT/β-catenin signaling pathways was reported to be mediated by the cytosolic β-catenin destruction complex, through regulatory interactions taking place in the cell nuclei (Heallen et al., 2011; Rosenbluh et al., 2012; Wang et al., 2014), or through other mechanisms (Azzolin et al., 2014; Cai et al., 2015; Imajo et al., 2012; Oudhoff et al., 2016; Park and Jeong, 2015). Recently, interactions between these two pathways were also shown to occur during epidermal homeostasis, and we and others showed that increased YAP activity in the basal keratinocytes of murine skin results in β-catenin activation (Akladios et al., 2017a, 2017b), and in severe epidermal hyperplasia in the interfollicular epidermis and in the hair follicles (Beverdam et al., 2013; Schlegelmilch et al., 2011; Zhang et al., 2011). However, the precise underlying molecular mechanism of how epidermal YAP activity promotes β-catenin activity to drive keratinocyte proliferation remains elusive.

In the present study, we found that inhibition of canonical Wnt/β-catenin signaling in the skin of YAP2-5SA-ΔC mice results in an amelioration of the epidermal hyperplasia in the interfollicular epidermis. In addition, we found that epidermal YAP promotes WNT16 expression in HaCaT keratinocytes and in the mouse interfollicular epidermis *in vivo*. Furthermore, we established that WNT16 promotes keratinocyte proliferation both independently of β-catenin, and through canonical WNT16/β-catenin signaling depending on the levels of WNT16 expression. We conclude that WNT16 is the WNT ligand that under normal physiological conditions promotes canonical WNT/β-catenin signaling to drive proliferation of keratinocytes in response to epidermal YAP activity *in vitro* and in the murine interfollicular epidermis *in vivo*.

2. Material and methods

2.1. Animals

All animal experimental procedures performed were conducted under protocols approved by the UNSW Australia's Animal Care and Ethics Committee Unit, and in compliance with the National Health and Medical Research Council Australian code of practice (8th edition, 2013). Thirty-eight days old YAP2-5SA-ΔC mice were topically treated with 100 μl of 5 μM XAV-939 (Chem-Supply, X0077-25MG) diluted in DMSO and vehicle solution (DMSO) daily for 13 days.

2.2. Tissue processing and histological and immunofluorescence staining

Full thickness mice skin tissues were fixed in 4% paraformaldehyde, paraffin embedded, sectioned, and histology-stained following routine procedures. Antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) and a Milestone RHS-1 Microwave at 110 °C for 5 min. Tissue sections were immunostained using previously standardized methods, and confocal images were captured using an Olympus FV1200 laser scanning confocal microscope. Epidermal thickness and immunosignal intensity were quantified using 3 mice and 3 skin regions per mouse. Immuno-signal was quantified in a semi-automated fashion using ImageJ software. Antibody information is available in Table S1.

2.3. Western blots and qRT-PCR analysis

Protein and RNA were isolated from full thickness mouse skin biopsies and HaCaT cells using TRIzol® reagent (Thermo Fisher Scientific, 15596026) following standard protocols. Protein lysates were analysed by western blot, intensity of bands was quantified with ImageJ software and normalized to GAPDH, GSK3β or β-catenin. Primary and secondary antibody information is available in Table S1. Quantitative RT–PCR assays were carried out using Fast SYBR® Green Master Mix (Thermo Fisher Scientific, 4385612) and Mx3000P qPCR System (Agilent Technologies), and were analysed by the comparative cycle time method, normalizing to *18S* ribosomal or *GAPDH* RNA levels. Human and mouse primer information is available in Table S2.

2.4. Cell lines and transfections

HaCaT immortalized keratinocytes of passages between 15 and 21 were maintained in DMEM/F-12 (Sigma, D8062), supplemented with 10% FBS (Gibco, 10437-028) and 1× Penicillin-Streptomycin (Gibco, 15140-122) in a 5% CO_2 incubator at 37 °C. Transient transfections were performed using Lipofectamine3000 (Thermo Fisher Scientific, L3000015) according to manufacturer's instructions. To overexpress dominant active YAP, pDsRed Monomer C1-YAP2-S127A mutant (Addgene, 19058) and pDsRed Monomer vector control DNA were used. WNT16 overexpression was carried out using pcDNA3.2-WNT16-V5 (Addgene, 35942) and pcDNA3.2 vector control. *YAP* knockdown was performed using MISSION® Universal and *YAP* siRNA (Sigma). *WNT16* knockdown was performed using MISSION® *GFP* and *WNT16* esiRNA (Sigma). MTT assays were performed using Thiazolyl Blue Tetrazolium Bromide (Sigma). Immunostaining assays were performed following standard protocols. Antibody information is available in Table S1. Imaging was performed using an Olympus FluoView™ FV1200 Confocal Microscope.

2.5. Statistical analysis

Statistical significance was determined by Student's unpaired *t*-tests. Error bars represent mean ± SEM. Asterisks indicate statistical significance, where $P < 0.05$ was used as significance cut-off.

3. Results

3.1. XAV939 inhibition of canonical WNT signaling ameliorates epidermal hyperplasia of YAP2-5SA-ΔC mice

We recently established that epidermal YAP activity promotes β-catenin activity to drive keratinocyte proliferation in the mouse skin *in vivo* (Akladios et al., 2017a,b). However, it remains unclear whether these interactions are mediated by paracrine activation of canonical Wnt signaling, or by other YAP/β-catenin regulatory interactions. Firstly, we assessed whether these were caused by activation of canonical Wnt/β-catenin signaling activity. To do so, we used YAP2-5SA-ΔC mice, which display increased epidermal YAP activity, increased β-catenin activity, and severe hyperplasia of the basal layer due to increased keratinocyte proliferation. Furthermore, the skin of these mice also display abnormal hair follicles and hyperkeratosis (Akladios et al., 2017a,b; Beverdam et al., 2013). We topically treated YAP2-5SA-ΔC mice with XAV-939 or vehicle daily for thirteen days. XAV-939 is a small molecule that selectively inhibits canonical (and not non-canonical) Wnt signaling through stabilizing Axin2 and promoting the destruction of cytosolic β-catenin (Distler et al., 2013).

Histological analysis showed that the thickness of the hyperplastic interfollicular epidermis of XAV-939-treated YAP2-5SA-ΔC mouse skin was approximately a third of that of vehicle-treated YAP2-5SA-ΔC mouse skin (Fig. 1c–d.1; $P < 0.04$, $N = 3$). Effective inhibition of Wnt/β-catenin signaling activity by XAV-939 was confirmed by reduced nuclear active β-catenin (Fig. 1f, f.1; $P < 0.002$, $N = 3$), and reduced nuclear localization of β-catenin direct target Cyclin D1 (Fig. 1h, h.1; $P < 0.0001$, $N = 3$) in the interfollicular epidermis. Furthermore, nuclear expression of the basal epidermal stem/progenitor cell marker P63 ($P < 0.0001$, $N = 3$) (Fig. 1i *vs.* j & j.1), and of proliferation marker Ki67 ($P < 0.008$, $N = 3$) (Fig. 1k *vs.* l & l.1) were also significantly reduced in the XAV-939- *vs.* vehicle-treated YAP2-5SA-ΔC interfollicular epidermis.