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Original Paper

Evidence For a Fibrogenic Interaction Between the Aryl Hydrocarbon Receptor and the Wnt/β-Catenin Pathways in Human Keratinocytes and Fibroblasts

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Key Words
Fibrosis • Systemic sclerosis • MMP1 • Collagen type I • Aryl hydrocarbon receptor

Abstract
Background/Aims: Systemic sclerosis (SSc) is a rare autoimmune and fibrotic disease, which often manifests in the skin. The aryl hydrocarbon receptor (AHR) is critical for skin homeostasis; however, little is known about its role in fibrosis and SSc. TGFβ, a known target gene of AHR and a fibrogenic cytokine, is also implicated in SSc. Wnt/β-catenin signaling promotes fibrosis, and both the TGFβ and Wnt/β-catenin pathways can act synergistically. Therefore, we investigated the potential triangular crosstalk between TGFβ, AHR, and Wnt/β-catenin signaling in fibrosis. ***Methods:*** Human dermal fibroblasts (HDF) and HaCaT keratinocytes—both "wild-type" and AHR-deficient—were cultured in mono- and co-cultures. Cells were treated with TGFβ, and the tryptophan photoproduct 6-formylindolo[3,2-*b*]carbazole (FICZ), an AHR agonist. Collagen type I (COL1A1) and matrix metalloproteinase-1 (MMP1) were quantified by ELISA, and Wnt/β-catenin pathway genes were analyzed using ddPCR. Cell migration was assessed using the scratch assay, and proteome profiling was performed for secreted factors. ***Results:*** AHR deletion in HDF reduced Wnt/β-catenin pathway gene expression, but adding an AHR ligand did not further increase the expression of these genes. AHR deficiency in HDF abrogated TGFβ-induced collagen production in monocultures, as did the presence of HaCaT cells in co-cultures. A proteome profile and KEGG analysis of co-cultures showed AHR-dependent regulation of immune-related genes. Finally, scratch closure was also AHR-dependent in both cell types, and this effect could not be fully rescued by TGFβ addition. ***Conclusions:*** This study highlights a context-dependent role of AHR in skin fibrosis and a complex triangular

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relationship with TGFβ and Wnt/β-catenin signaling. More research is needed to evaluate AHR as a potential therapeutic target in SSc.

Introduction

Fibrosis is a severe pathological process characterized by excessive extracellular matrix (ECM) deposition, most commonly affecting the skin, lungs, and gastrointestinal tract. Systemic sclerosis (SSc)-is an autoimmune connective tissue disease characterized by fibrosis of multiple organs, including the skin. SSc is a debilitating disease, and therapeutic options remain limited partly due to the disease's complexity, but also because of the gaps in knowledge regarding the underlying molecular biology. Several major fibrogenic players are known, in particular TGFβ and Wnt/β-catenin signaling, which can regulate the balance of collagen secretion and degradation. Less considered is the aryl hydrocarbon receptor (AHR) as an inducible transcription factor that possibly links to TGFβ and fibrosis [1, 2]. In the skin, crosstalk between keratinocytes and fibroblasts is important for maintaining skin homeostasis [3–5]. Fibroblasts secrete and provide extracellular matrix (ECM) molecules, i.e. collagens, which in turn are degraded by matrix metalloproteinases (MMPs), balancing and structuring the ECM. In connective tissue diseases, fibroblasts produce excess ECM and adhere more to it, leading to increased tissue stiffness [6]. MMP levels are regulated by endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) [7], and ECM seems to depend on keratinocyte-derived cytokines, such as IL-1β [3, 5].

A key player in ECM homeostasis is transforming growth factor (TGF)-β, which can aberrantly activate fibroblasts to produce abundant collagen. TGFβ is produced mainly by fibroblasts and immune cells in the skin. Indeed, a simple and recognized *in vitro* fibrosis model is the addition of TGFβ to fibroblasts, which vastly enhances collagen production [8]. However, it is also recognized that Wnt/β -catenin signaling pathway contributes to pathological fibrosis [9, 10]. As the starting point of the signaling, Wnt binds to the surface receptor frizzled, which stops the constantly ongoing degradation of cytoplasmic β-catenin and allows its translocation to the nucleus. There, β-catenin acts as a transcriptional co-factor for various target genes, including fibrogenic genes such as collagen [11, 12]. Wnt signaling can be inhibited by, e.g., WIF1 or DKK2 upregulation, which prevents β-catenin stabilization in the cytoplasm [13]. Interestingly, TGFβ modulates Wnt/ β-catenin signaling at various points, promoting both Wnt production and enhancing β-catenin activation [14].

The AHR is known to be involved in skin physiology [15, 16]. Interestingly, skin fibroblasts from patients with SSc have been reported to exhibit reduced AHR levels, and the authors thus suggested a potential anti-fibrotic role of the AHR [17]. Murai et al. (2018) demonstrated in normal human dermal fibroblasts that the up-regulation of the ECM degrading MMP1 enzyme may occur in an AHR-dependent manner [7], which was also found in orbital fibroblasts [18]. While increased MMP1 levels have been observed in other autoimmune conditions such as rheumatoid arthritis [19], biopsies from SSc patients have also shown decreased MMP1 [20, 21], showing disease-specific regulation.

Mechanistically, TGFβ can suppress AHR transcription, while the AHR can modulate TGFβ signaling via interactions with key regulatory proteins. The AHR is implicated in the Wnt/β-catenin pathway as well [22–25], because apparently AHR activation antagonizes Wnt/β-catenin signaling [26–28]. While AHR suppresses pro-fibrotic behavior in fibroblasts, and robust anti-Wnt mechanisms are established in other cells, a direct and functionally verified connection between AHR and Wnt/β-catenin signaling in mammalian fibroblasts remains to be demonstrated.

In this study, we therefore investigate a potential triangular crosstalk between the Wnt/β-catenin, AHR, and TGFβ signaling pathways in human dermal fibroblasts and keratinocytes using AHR-deficient cells. Additionally, we examine keratinocyte-fibroblast interactions under fibrotic conditions using *in vitro* co-culture models.

Materials and Methods

Cell Monoculture

We used HaCaT cells, a spontaneously immortalized, non-tumorigenic human keratinocyte line [29], and human dermal fibroblasts (HDF). AHR was deleted in HaCaT by CRISPR/Cas9 in the IUF's in-house facility, described previously [30], to generate AHR-KO cells. HDF and an AHR-deficient clone thereof, DU96 (referred to as HDF-KO in this manuscript), were cultured at 37°C and 5% CO_2 in DMEM medium (Pan Biotech, cat. no. *P04-03590*) supplemented with 10% FBS (Pan Biotech, cat. no. *P40-37500*) and antibiotics (penicillin and streptomycin). Cells were seeded in six-well plates at a density of $2x10^5$ cells per well in 1 ml. When the cells reached 70% confluence, they were treated with reagents and incubated for 24h and 48h. Similarly, HaCaT cells were seeded at a density of $5x10^5$ cells per well and cultured until they reached 70% confluence in DMEM medium as described above. After reaching confluence, both HDF and HaCaT cells were treated with 100 nM 6-formylindolo[3, 2-*b*]carbazole (FICZ, MedChem Express LLC, USA) or TGFβ (Gibco, cat. no. *PHG9214*) at a concentration of 10 ng/ml or 25 ng/ml, and incubated for 24 h and 48 h. Cell pellets were preserved in 500 µl of RNAlater (Invitrogen, cat no. *AM7022*) and stored at -80°C for further analysis. Supernatants from the monoculture experiment were collected for subsequent ELISA tests. The number of replicates is indicated in the figure legends.

Co-cultures setup

HDF and HaCaT cells (and their respective AHR-KO variant) were co-cultured using a transwell insert system with a 0.4 µm pore diameter (Merck, cat. no. *PTHT06H48*). HDF cells were seeded at the bottom of six-well plates at a density of 2×10^5 cells per well, while HaCaT cells were independently seeded onto hanging cell culture inserts at a density of 5×10^5 cells per insert to allow for confluence. Cells were incubated at standard conditions until reaching approximately 70% confluence. Upon reaching the desired confluence (after approx. 24 h), the inserts containing either HaCaT or HaCaT-KO cells were carefully transferred into wells containing the corresponding HDF populations to establish the co-culture and incubated for 48 h in standard conditions. This resulted in two experimental groups, consisting of HDF and HaCaT cells with AHR or their AHR impaired variant. All conditions were performed in triplicate. Supernatants from the co-culture experiment were collected for subsequent ELISA (MMP1 and COL1A1) and Proteome Profiler analyses.

COL1A1 and MMP1 measurement by ELISA

Commercial ELISA kits were used to quantify collagen type I (RayBiotech, cat. no. *ELH-COL1A1*) and MMP1 in cell culture supernatants (RayBiotech, cat. no. *ELH-MMP1*). Tests were performed according to the manufacturer's instructions. All samples were analyzed in triplicate.

Gene expression

Gene expression analysis was conducted on cultured cells from monoculture experiments only. RNA from ~2 $x10^5$ HDF and ~ 1 $x10^6$ HaCaT cells was isolated using RNeasy Micro RNA Kit (Qiagen, cat. no. *74004*). The quantity and quality of isolated RNA were evaluated on a DeNovix spectrophotometer. RNA purity was determined based on the 260/280 nm OD ratio, with expected values between 1.8 and 2.0. RNA was stored in ultra-clean, RNase-free water provided by the manufacturer at -80°C until further analysis.

Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions. ddPCR Supermix for Probes (no dUTP; Bio-Rad, Hercules, CA) and TaqMan probes (Thermo Fisher Scientific, USA) were used for the gene expression analysis: *AHR* (Hs00169233_m1), *FZD2* (Hs00361432_s1), *DKK2* (Hs00205294_m1), *TCF7* (Hs01556515_m1), *LEF1* (*TCF10*, Hs01547250_m1), *WIF1* (Hs00183662_m1), and *CTNNB1* (Hs00164383_m1). These genes, except *AHR*, are hereafter referred to as "Wnt/β-catenin pathway genes". Each reaction was then loaded into the sample well of an eight-well disposable cartridge (Bio-Rad) along with 70 µl of droplet generation oil (Bio-Rad), and droplets were formed in a final volume of 40 µl using the Droplet Generator (Bio-Rad). Droplets were transferred to a 96-well PCR plate, heat-sealed with foil, and amplified using a C1000 Touch Thermal Cycler (Bio-Rad). Results expressed in copies/µl were converted into copies/ng. Gene expression analysis was performed in three independent experiments, with each sample analyzed

in triplicate. To determine if the incubation time (24 or 48 hours) influenced the effect of other predictive variables on gene expression, regression models incorporating an interaction term between time and these variables were employed. Although a subset of samples showed higher gene expression after 48 hours of incubation compared to 24 hours, the regression model did not reveal any significant interaction between time and the effect of other variables ($p > 0.05$, data not shown). Furthermore, interaction plots visually confirmed the absence of such an effect. This result indicates that the incubation time did not significantly modulate the impact of the other variables on gene expression. Consequently, results for both incubation times are presented together in the final gene expression figures and graphs.

Proteome profiler

For the analysis of proteins secreted by HDF and HaCaT in co-cultures, the Proteome Profiler Human XL Cytokine Kit (cat. no. *ARY022B*, R&D Systems, USA) was used. A total of 500 µl of supernatant from the WT/WT co-culture and 500 µl from the KO/KO co-culture were analyzed according to the manufacturer's protocol. Specifically, antibody-labeled membranes provided by the manufacturer were incubated in a kit-provided buffer for one hour at room temperature. After removing the buffer, the supernatants - diluted in the same buffer - were added and incubated overnight at 2–8°C. The following day, the protocol was continued as per the manufacturer's instructions, resulting in membranes with bound proteins. Chemiluminescence analysis was performed using the iBright CL750 imaging system (Thermo Fisher Scientific, USA), with the membrane exposure time automatically set by the device software to approximately 30 minutes. Signal intensities were visually scored using a 4-point scale: 0 = no expression, 1 = weak (+), 2 = moderate (++), and 3 = strong (+++). Differences in signal intensity between KO/KO and WT/WT groups were calculated and visualized as (KO/KO – WT/WT) (Table S2). To validate the findings obtained from the Proteome Profiler, we further quantified IL-8 secretion using an independent ELISA assay (cat. no. *KE00006*, Proteintech) according to manufacturer's instructions.

Proteins showing differential expression were subjected to KEGG pathway enrichment analysis using the clusterProfiler R package [31] and the org.Hs.eg.db annotation database [32], in order to identify biological pathways most affected by AHR deletion. Data visualization, including signal-intensity plot, was performed using the ggplot2 package [33] in R. Pathways were categorized based on the number of upregulated (positive signal difference) and downregulated (negative signal difference) proteins in KO/KO cells compared to WT/WT.

Scratch assay

Cells were seeded in 24-well culture plates at a high density: $1x10^5$ cells for HaCaT and $8 x10^4$ cells for HDFs and cultured under standard conditions. After overnight incubation, when the cells reached 95% confluence, the cell layers were scratched diagonally in the middle of each well using a 200 µl pipette tip. After scratching, cells were washed with PBS four times to remove the debris formed by scratching. Cells were treated with TGFβ 25 ng/ml, FICZ 100 nM, or a combination of TGFβ 25 ng/ml + FICZ 100 nM. In the case of HDF cells (Fig. 6C), additional conditions included treatment with the AHR inhibitor CH223191 (10 µM), either alone or in combination with TGFβ. Images were taken at 0 h, 24 h and 30 h or 48 h after scratching. To assess the effects of AHR impairment in HDF cells, we used the AHR antagonist CH223191 to block AHR signaling, as HDF-KO cells were no longer available for these experiments. Serum-free DMEM medium was changed daily with the appropriate treatment. Light microscope images with a 40x magnification were taken right after scratching, as well as after 20 h and 30 h of incubation in case of HDF cell and 24 h and 48 h in the case of HaCaT cells. The images were then analyzed using ImageJ wound healing size tool, an automated option which, after manual corrections, allowed the measurement of the area of the wound gap in percentage of the whole photographed area.

Statistical analysis of gene expression data

Statistical analyses and data visualizations were performed in the R environment (R Core Team, 2024). The following R packages were used: tidyverse, readxl, ggpubr, cowplot, flexplot, and GGally. To assess the effect of incubation time and its interactions with other variables on gene expression, a generalized regression model was applied. Correlation analysis was performed on logarithmically transformed gene expression data using the ggpairs function from the GGally R package. The results were presented as a correlation plot matrix, including both visual representations of the correlations and numerical values of

the correlation coefficients with their corresponding p-values. Comparisons of expression levels between groups were conducted using non-parametric statistical tests: the Wilcoxon test for pairwise comparisons and the Kruskal-Wallis test for multiple group comparisons. Due to the exploratory nature of the study, correction for multiple testing was not applied. The aim was to identify potential patterns and hypotheses for future validation rather than to draw definitive inferential conclusions. A p-value < 0.05 was considered statistically significant.

Statistical analysis of ELISA data

GraphPad Prism software (version 10.1.2; GraphPad Software, San Diego, CA, USA) was used for analysis and data visualization. Two-way ANOVA followed by a post hoc multiple comparisons test was used to analyze differences between groups. A p-value < 0.05 was considered statistically significant.

Results

Absence of AHR modulates gene expression of Wnt/β-catenin genes – downregulation in fibroblasts, upregulation in HaCaT

We performed digital droplet PCR with RNA isolated from AHR-proficient and AHR-deleted HDF and HaCaT cells to test whether AHR affects the expression levels of the Wnt/β-catenin pathway genes frizzled2 (*FZD2*), Wnt inhibitory factor (*WIF1*), dickkopf inhibitor 2 (*DKK2*), lymphoid enhancer binding factor 1 (*LEF1*), transcription factor 7 (*TCF7*), and β-catenin 1 (*CTNNB1*).

As shown in Fig. 1A and supplementary Figure S1, the absence of AHR in fibroblasts resulted in a significantly lower expression of all investigated Wnt/β-catenin pathway genes. *WIF1* and *LEF1* transcript levels were below the limit of detection and are therefore not shown. However, DNA methylation analysis for these genes is provided in the supplementary material, suggesting that epigenetic regulation may contribute to their silencing (Figure S2-S5). Except for *DKK2* expression, AHR deficiency in HaCaT cells resulted in upregulation of these pathway genes, suggesting that AHR suppresses their expression (Fig. 1B). Again, *WIF1* and *LEF1* levels were below the limit of detection, irrespective of the presence or absence of AHR.

Of note, the overall expression levels of *FZD2* and *CTNNB1* were similar in both cell types. Notably, *DKK2* expression was higher in both WT cell lines. Together, the data indicate for the first time that the presence of AHR is needed for basal expression of selected Wnt/β-catenin pathway genes and β-catenin expression itself in HDFs. In contrast, presence of AHR appears to have a dampening effect on the expression of these genes in HaCaT cells.

AHR activation did not affect Wnt/β-catenin pathway gene expression

Albeit it is an inducible transcription factor, the AHR has a basal activation level through ligands present in normal media (tryptophan derivatives). *In vivo*, dietary and microbial ligands, and UVB-generated ligands, especially the tryptophan dimer FICZ, are relevant for the skin. Considering (i) that SSc manifests in the skin very often and (ii) the results described above, we asked how AHR activation might affect the expression of Wnt/β-catenin pathway genes. We therefore incubated HDF cells with FICZ and determined the expression levels of the Wnt/β-catenin pathway genes (Fig. 1A). Surprisingly, the addition of FICZ to HDF cells had no significant effects, in contrast to the marked changes observed in AHR-deficient HDF (both shown in Fig. 1A). Thus, ligand-mediated AHR activation did not alter the expression levels of the selected Wnt/β-catenin pathway genes in either HDF or HaCaT cells (Fig. 1A and 1B).

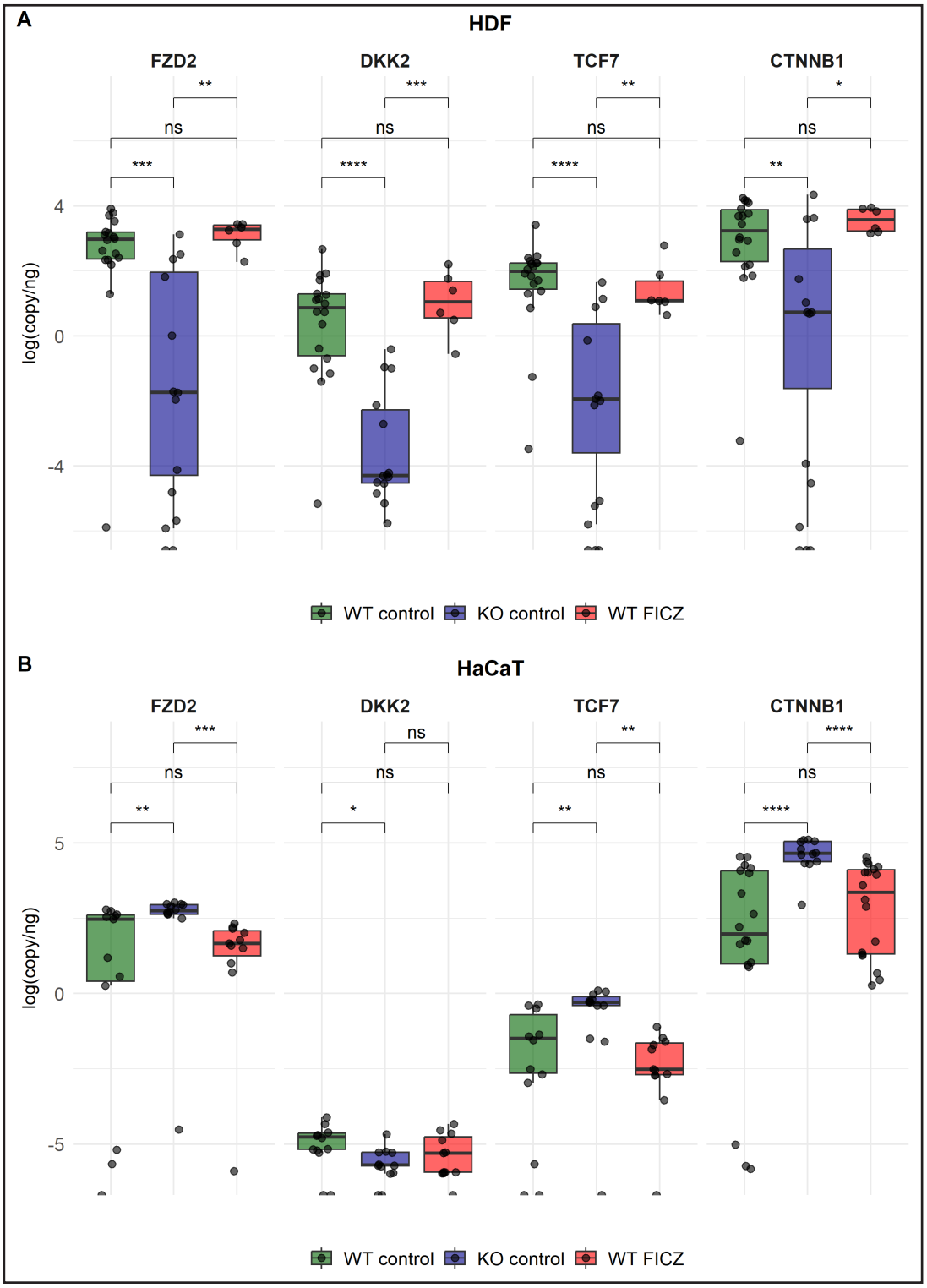

Fig. 1. Expression of selected Wnt/β-catenin pathway genes measured by ddPCR, presented as log-transformed copies/ng in human dermal fibroblasts (HDF, panel A) and HaCaT, (panel B). Green, blue, and red boxplots with individual data points represent "wild-type" (WT), AHR-knockout (KO) cells, and WT cells treated with the AHR agonist FICZ (red), respectively. Cells were collected from three independent experiments, and ddPCR results were pooled from two timepoints (24 h and 48 h). All samples were performed in triplicate. Statistical comparisons were performed using the Wilcoxon test. *: p <= 0.05; **: p <= 0.01; ***: p <= 0.001; ****: p <= 0.0001; ns = not significant.

TGFβ effects on Wnt/β-catenin genes and β-catenin in AHR-deficient or -proficient HDF and HaCaT cells

The addition of TGFβ to cultured fibroblasts is a recognized, simple fibrosis model [8], and canonical Wnt/β-catenin signalling can be necessary for TGFβ-mediated fibrosis [34]. We therefore asked if the Wnt/β-catenin pathway genes are modulated not only by AHR, but also by TGFβ in HDF and HaCaT cells. Hence, we added TGFβ (10 ng/ml or 25 ng/ml) to the cultivated cells for up to 48 hours, and afterwards performed ddPCR for the Wnt/β-catenin pathway genes mentioned above. We also measured *AHR* expression itself to determine if it is changed by TGFβ addition. In HDF cells, with the exception of *FZD2*, addition of TGFβ to the culture medium reduced expression levels compared to the control (Fig. 2A). In the HDF-KO cells, significantly more *FZD2* and *CTNNB1* mRNAs were detectable after TGFβ exposure, but only at the higher TGFβ dose (Fig. 2B). In HaCaT cells, TGFβ addition did not affect any of the pathway genes, except for an increased expression of *CTNNB1* (Fig. 2C). In HaCaT-KO cells, loss of AHR resulted in lower expression of FZD2 and β-catenin genes when TGFβ was added to cultures, even lower than the WT/KO decrease (Fig. 2D). The key observation is that TGFβ treatment led to opposite effects on *CTNNB1* expression in the two skin cell types: it decreased *CTNNB1* mRNA levels in HDF WT cells, while increasing them in HaCaT cells. Curiously, in HaCaT-KO cells, the combination "loss of AHR plus TGFβ addition" resulted in reduced Wnt/β-catenin pathway gene expression.

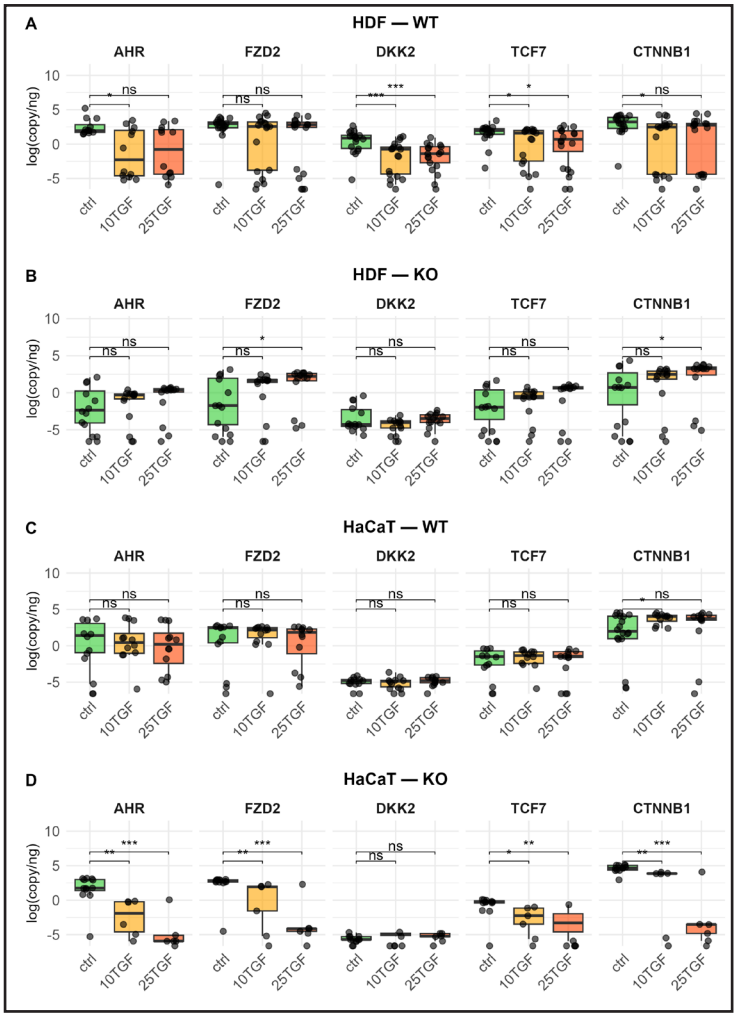

Fig. 2. Effect of TGFβ treatment on the expression of selected Wnt/β-catenin pathway genes, measured by ddPCR, in "wild-type" (WT) and AHR-deficient (KO) human dermal fibroblasts (HDF, panels A and B) and HaCaT (panels C and D). Cells were treated with TGFβ at 10 ng/mL (10TGF) or 25 ng/mL (25TGF) for 24 h and 48 h. Cells were collected from three independent experiments. Gene expression results from different timepoints were pooled and presented as log-transformed copies/ng. Boxplots with individual data points are shown. Control samples are shown in light green, while TGFβ-treated samples are shown in yellow (10TGF) and orange (25TGF). All samples were performed in triplicate. Statistical comparisons were performed using the Wilcoxon test. *: p <= 0.05; **: p <= 0.01; ***: p <= 0.001; ****: p <= 0.0001; ns = not significant.

AHR deficiency in HDF abrogates TGFβ-induced collagen production in monocultures, as does the presence of HaCaT cells in co-cultures

Collagen 1A1 (COL1A1), one of the main components of the ECM, often dysregulated in fibrosis, is inducible upon TGFβ treatment in fibroblast culture [35]. We treated our cells with TGFβ and assessed COL1A1 protein production by ELISA. As expected, HDF cells secreted more COL1A1 upon TGFβ treatment (Fig. 3A, orange bars), already at 24 hours (with 25 ng/ml) and then further at 48 hours later (detectable for both 10 ng/ml and 25 ng/ml T TGFβ). In contrast, in HDF-KO cells, COL1A1 secretion was lower than in HDF (about 50%), and only a very moderate increase was detectable after TGFβ addition (Fig. 3A, orange bars). Surprisingly, addition of FICZ inhibited TGFβ-mediated collagen production in HDF cells, and somewhat enhanced it in HDF-KO cells, especially at 48 hours. Nonetheless, absolute levels remained lower than in control HDF or HDF-KO cells (Fig. 3A, green bars). Thus, in HDF cells, collagen production regulation involves AHR signaling as well as TGFβ signaling. Given the evidence in the literature that keratinocytes produce factors which influence collagen production by fibroblasts [3, 36], we next asked whether and how co-culturing HaCaT with HDF could affect collagen production by the latter (Fig. 3B). HaCaT were placed in the upper chamber inserted into a well and HDF were placed at the bottom of the well, so that soluble products (less than 1000 kDa) could reach the lower well. As shown in Fig. 3B, co-culturing AHR-proficient HaCaT and HDF (labeled WT co-culture in the figure) for 48 hours resulted in a collagen production of 2741.85 ± 7.46 ng/ml in the medium. Addition of TGFβ to medium decreased collagen significantly by nearly 50%, to about 1465.38 ± 137.85 ng/ml. When AHR was absent in both cell types (labelled as KO co-culture), the co-cultures´ supernatants yielded lower collagen than in co-cultured WT cells. This likely reflects that HDF-KO are low in collagen production anyway (Fig. 3A), and the presence of HaCaT-KO cells could not rescue this. Importantly, co-cultivation of HaCaT cells with HDF cells (whether WT/WT or KO/KO) suppressed collagen production by TGFβ (presumably by the HDF cells), but otherwise presence or absence of AHR in the co-cultured HaCaT did not positively or negatively influence collagen production.

MMP1 secretion by TGFβ addition is dependent on AHR

MMP1 production is important for degrading collagen and thus regulating ECM. Excessive collagen deposition due to a lack of degradation significantly adds to the fibrosis process. HDF cells produced robust amounts of MMP1, but TGFβ-treatment almost halved this over 24/48 hours of cultivation (Fig. 4A), and MMP1 production was entirely lost in AHR-deficient HDF. Highlighting the role of AHR in this, the treatment of HDF with FICZ rescued the TGFβ-mediated down-regulation and HDF-KO almost stopped secreting MMP1 upon FICZ addition. Although keratinocytes were described to produce MMP1 as well [37], in our hands HaCaT cells were very low producers, and TGFβ addition lowered secretion even further. AHR-deficient HaCaT were non-producers (Fig. 4B). We conclude that HaCaT cells contribute much less to MMP1-mediated collagen degradation than fibroblasts. Co-culturing HaCaT and HDF (both AHR-proficient) did not lessen the TGFβ-induced decrease of MMP1 production (Fig. 4C compare Fig. 4A and 4B). Congruent with the data of monocultures, MMP1 production was almost entirely lost in HDF KO cells, and the presence of HaCaT cells in the cultures did not change this. Taken together, AHR plays a crucial role in MMP1 production by HDF.

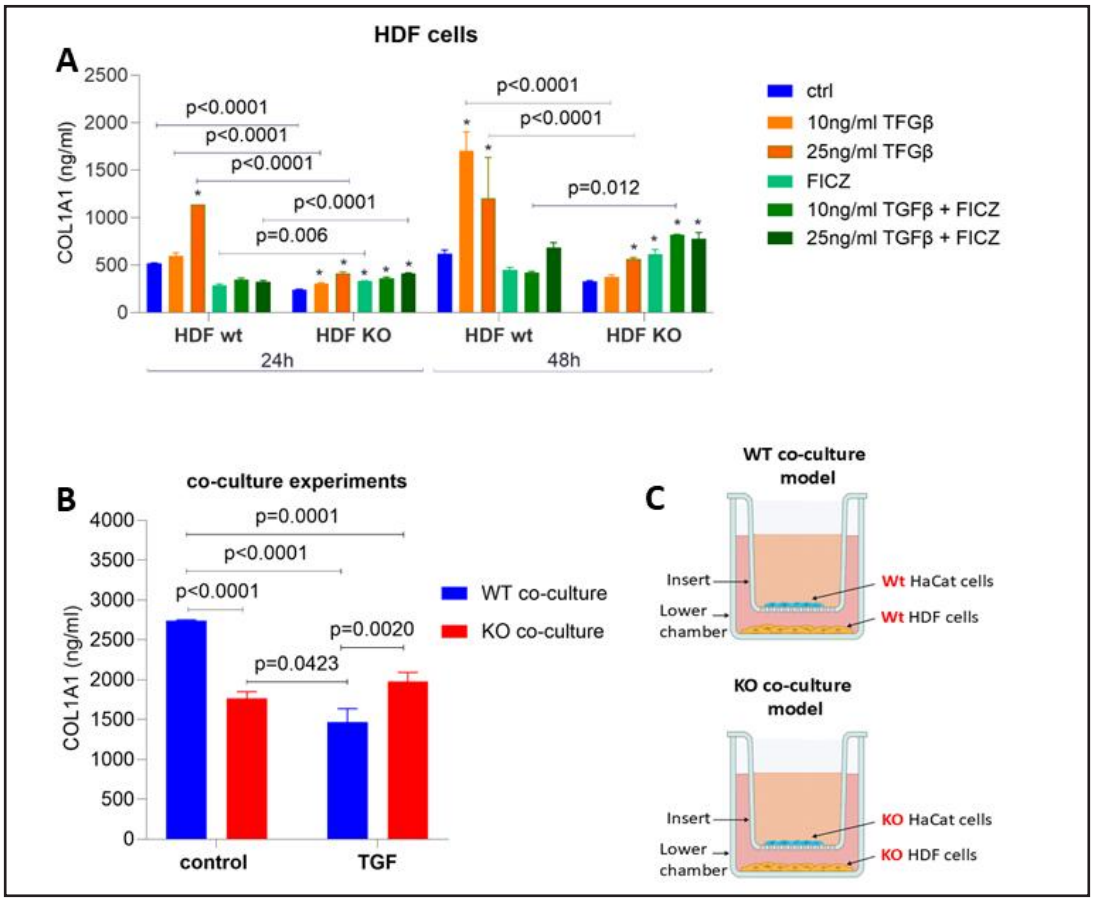

Fig. 3. COL1A1 secretion (ng/ml) by HDF cells (A) and HDF-HaCaT co-culture (B). (A) COL1A1 concentrations in HDF and HDF-KO cells after treatment with TGFβ (10 ng/ml, 25 ng/ml), FICZ, and their combinations for 24 and 48 hours. Control (untreated) samples are also shown. (B) COL1A1 secretion in co-cultures of HDF and HaCaT cells (wild-type (wt) and KO) after treatment with TGFβ (25 ng/ml) for 48 hours.(C) Schematic representation of the co-culture setup: the "wild-type" WT model consists of "wild-type" HDF and HaCaT cells, while the KO model includes AHR-deficient HDF and HaCaT cells. HDF cells were seeded in the lower chamber, and HaCaT cells were cultured on an insert. All samples were performed in triplicate. Two-way ANOVA. Bar plot. Mean ± SD. Post hoc multiple comparisons. $p \leq 0.05$. * indicates statistically significant difference in comparison to the corresponding untreated control.

Proteome profiler in co-culture

So far, we identified AHR to be involved in the expression/production of β-catenin, collagen, and MMP1, in both mono- and co-cultures. To further unravel the role of AHR, we next used a proteome profiler to compare protein secretion in the supernatants of WT/WT and KO/KO co-cultures (Supplementary material Table S2). We observed that vitamin D binding protein (vitamin D BP), retinol-binding protein 4 (RBP-4), platelet factor 4 (PF4, CXCL4), angiogenin, and adiponectin were the most upregulated proteins, whereas IL-8 (CXCL8) and monocyte chemoattractant protein-1 (MCP-1) exhibited the most pronounced downregulation in the KO/KO co-culture model compared to WT/WT (Fig. 5). To validate the downregulation of IL-8 observed in the proteome profiler, we performed an independent ELISA assay, which confirmed a significantly reduced IL-8 secretion in KO/KO co-cultures (Figure S6, Supplementary material). The analyzed proteome profiler indicated a loss of specific protein signals in KO co-cultures within pathways related to rheumatoid arthritis, cytokine-receptor interactions, and TNF signaling. These alterations in signaling pathways between the experimental co-culture models highlight the impact of AHR on inflammatory processes and immune-related signaling cascades.

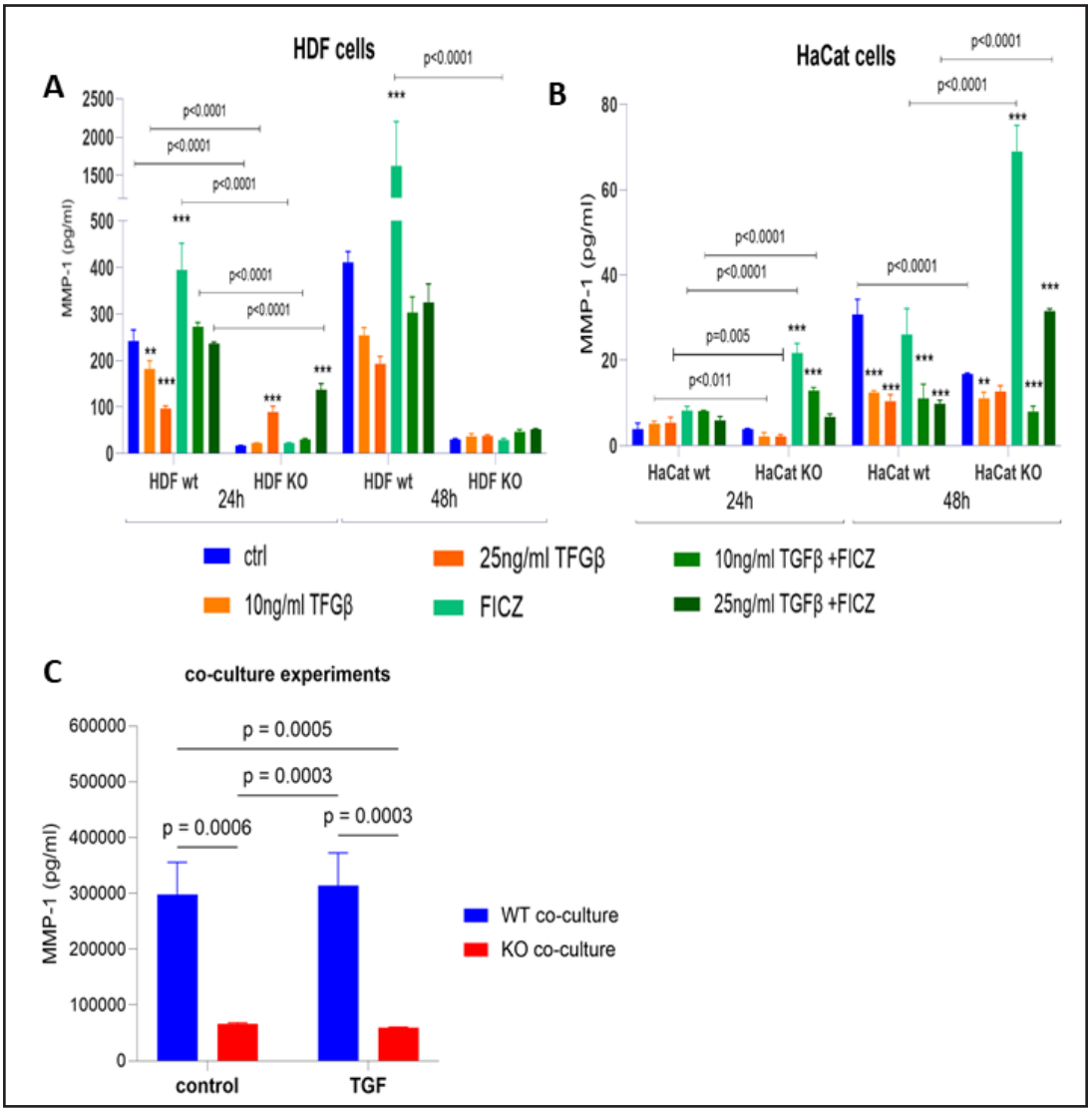

Fig. 4. MMP-1 secretion (pg/ml) in HDF and HaCaT cells cultured in monoculture (A, B) and co-culture (C). (A) MMP-1 concentrations in HaCaT WT and AHR KO cells after treatment with TGFβ (10 ng/ml, 25 ng/ml), FICZ, and their combinations for 24 and 48 hours. Control (untreated) samples are also shown. (B) MMP-1 concentrations in HDF WT and AHR KO cells under the same conditions as in (A). (C) MMP-1 secretion in co-culture of HDF and HaCaT (WT and AHR KO) after treatment with TGFβ (25 ng/ml) for 48 hours. HDF cells were seeded at the bottom of the well, while HaCaT cells were cultured on the inserts, allowing indirect interaction between the two cell types. All samples were performed in triplicate. Two-way ANOVA. Bar plot. Mean ± SD. Post hoc multiple comparisons. $p \leq 0.05$.

AHR deficiency impaired cells motility

Impaired wound healing can be found in SSc and a majority of patients experience digital ulceration at some point. We therefore assessed the relevance of AHR for the ability of HaCaT and HDF cells to close a gap in full cell layers, as an albeit limited token of two fibrosis-relevant functions: cell migration and wound healing. We introduced a gap with a pipette tip and microscopically quantified the area closed by immigrating cells 20/30 hours (HDF) and 24/48 hours (HaCaT) later. Proliferation was not specifically quantified, so its contribution cannot be considered separately. Fig. 6A-C shows the results for HaCaT, HaCaT-KO, HDF, or HDF cells treated with a chemical inhibitor of AHR signaling, CH223191, following treatment

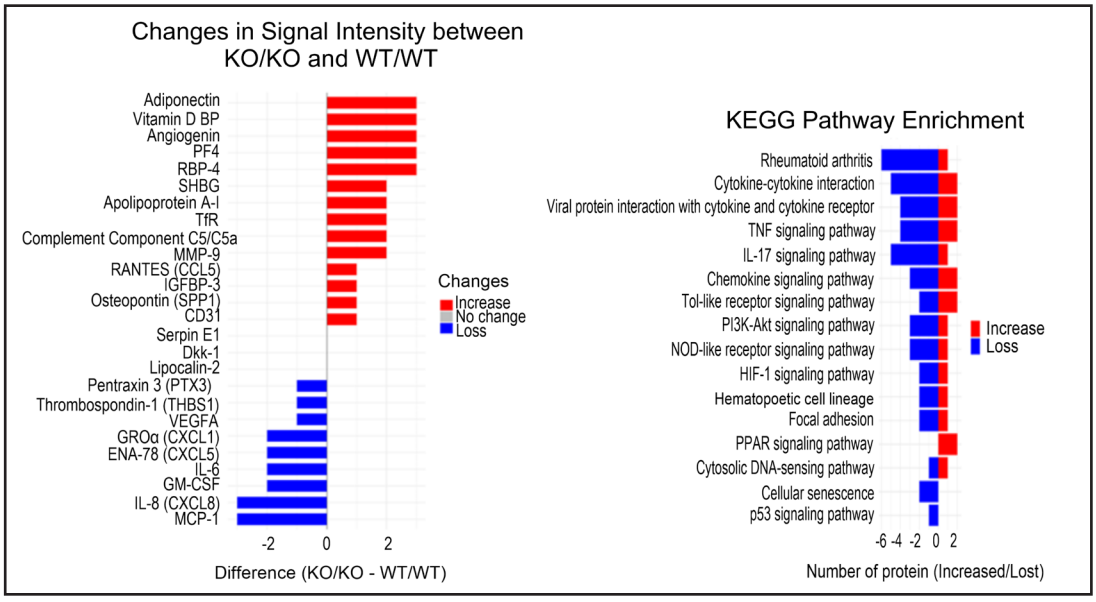

Fig. 5. Differential protein expression and KEGG pathway enrichment between KO/KO and WT/WT cell cultures. Left panel - changes in protein signal intensity between KO/KO and WT/WT groups measured in the supernatant of cell cultures. Signal intensity was visually assessed and semi-quantitatively scored on a 4-point scale: 0 = no expression, 1 = weak (+), 2 = moderate (++), 3 = strong (+++). The x-axis shows the difference in signal (KO/KO – WT/WT), with positive values (blue) indicating increased signal and negative values (red) indicating decreased or absent signal in KO/KO cells. Red bars indicate upregulated proteins, while blue bars represent downregulated/loss proteins in the KO/KO group relative to WT/WT. Right panel - KEGG pathway enrichment analysis. The x-axis represents the number of proteins per pathway that showed increased (red) or decreased (blue) signal in KO/KO compared to WT/WT samples, highlighting the biological pathways most affected by AHR deletion.

with TGFβ, FICZ, or a combination of both. While HaCaT cells were capable of slowly closing this "wound", the addition of FICZ or TGFβ (or both) vastly enhanced this process, with FICZ plus TGFβ resulting in full closure 48 hours after scratching (Fig. 6A). In contrast, HaCaT-KO were very slow in re-filling the scratch area, and only TGFβ addition (or TGFβ plus FICZ), but not FICZ alone, supported full closure (Fig. 6B, 6D). Thus, AHR-mediated TGFβ signaling is a major factor in the gap-closing capacity of HaCaT cells, and our data with HaCaT-KO/HDF plus chemical inhibition of AHR activity suggests that AHR signaling modulates TGFβ-induced changes in cell migration.

As expected, the scratch wound assay revealed that HDF are much better at migration than HaCaT cells (Fig. 6E), indeed, the scratch had closed at least 70% by 48 hours (compared to 30% in HaCaT). Again, the addition of FICZ and/or TGFβ accelerated the migration, while inhibition of AHR signaling slowed it down.

Discussion

Systemic sclerosis (SSc) is characterized by dysregulated collagen synthesis and degradation, accompanied by aberrant immune signaling. Important signaling pathways are TGFβ and/or Wnt/β-catenin. Here, we present data indicating that the aryl hydrocarbon receptor (AHR) is also involved in these processes. In this study, we employed (i) a simple fibrosis model by adding TGFβ to HDF and (ii) used AHR-deficient HDF and HaCaT cells to assess a possible additional impact of the AHR. Our study showed that the AHR had a significant role in maintaining cell functionality, including the response to fibrotic agents, particularly TGFβ, and collagen production.

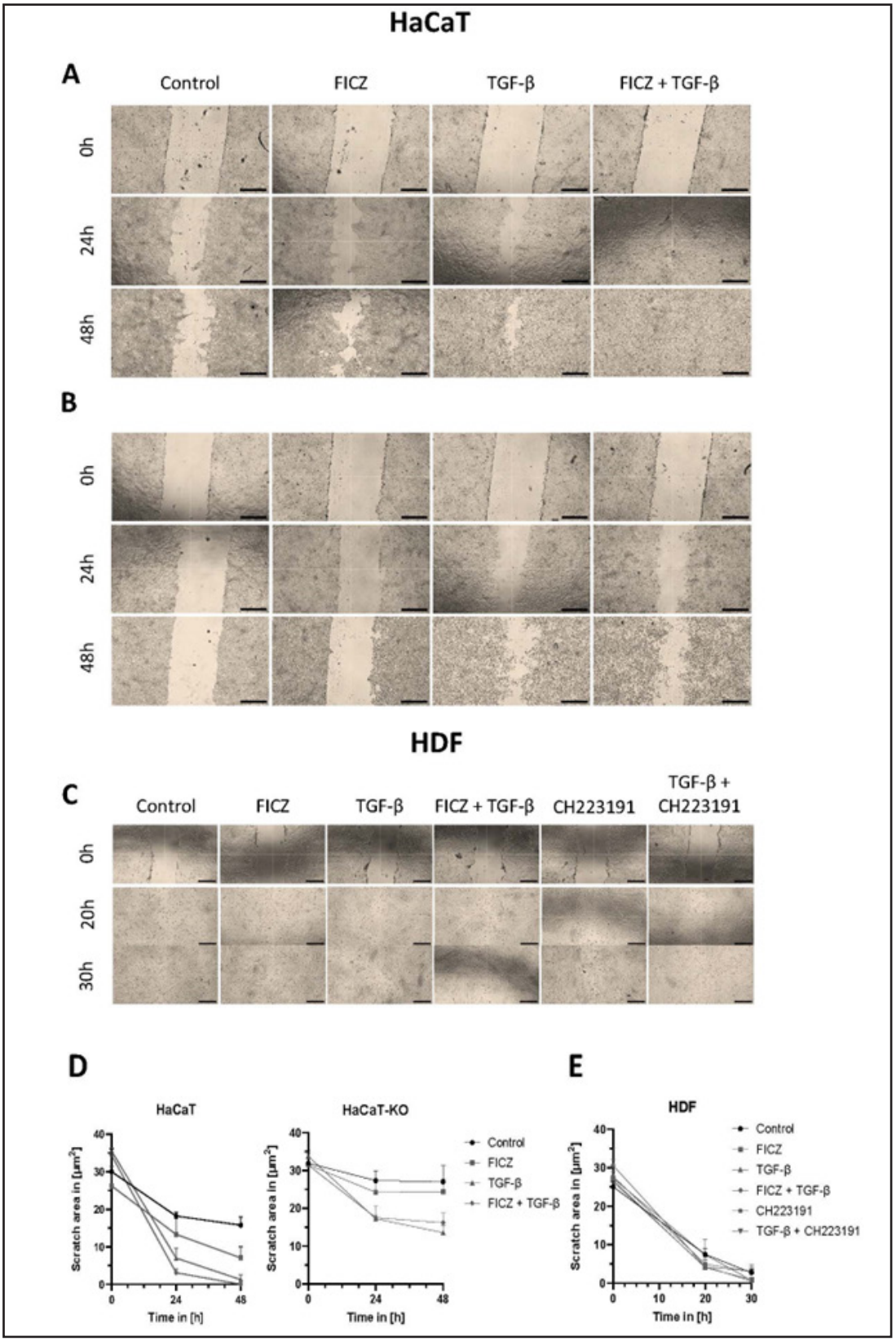

Fig. 6. Cell migration during wound healing in HaCaT (A), HaCaT-KO (B), and HDF (C) cells. Representative transmitted light microscopy images from scratch assays performed using a 200 µL pipette tip. Cells were treated with TGFβ (25 ng/mL), FICZ (100 nM), or a combination of both. For HDF cells (C), additional conditions included treatment with the AHR inhibitor CH223191 (10 µM), either alone or in combination with TGFβ. Images were acquired at 0, 24, and 30 or 48 hours post-scratch. Quantitative analysis of wound closure over time is shown for HaCaT and HaCaT-KO cells in panel D, and for HDF cells in panel E; data are presented as mean ± standard deviation (s.d.). Scale: 40× magnification; scale bar, 500 µm.

Wnt/β-catenin and AHR

Regarding Wnt/β-catenin signaling, an important player in SSc, we identified an influence of the AHR on β-catenin mRNA expression as well as on genes of its upstream signaling pathway. In AHR-deficient HDF cells, we observed lower levels of β-catenin mRNA compared to AHR-proficient cells, whereas in HaCaT cells, the opposite trend was observed. Given the known cell-specificity of the AHR, this finding was not unexpected. While recent studies have highlighted potential crosstalk between the AHR and Wnt/β-catenin signaling, no curated pathways in professional academic databases currently establish a direct mechanistic links between AHR, Wnt/β-catenin, (or TGFβ) signaling. One study showed by co-immunoprecipitation and immunofluorescence assays that the AHR associates with E-cadherin and β-catenin in normal murine mammary gland cells (NMuMG), suggesting its involvement in cell-cell adhesion [38]. Our study provided preliminary evidence indicating that AHR status significantly influences the regulation of the Wnt/β-catenin pathway in a cell type-dependent manner (HDF vs. HaCaT). Our observations align with the evidence that β-catenin functions primarily in transcriptional regulation in fibroblasts but is sequestered at adherens junctions in keratinocytes [39]. Available studies generally describe that the absence of AHR is associated with increased activity of the Wnt/β-catenin pathway, including elevated β-catenin levels [26–28, 40]. While this trend is observed in many tissues, exceptions do exist [41, 42], suggesting a more nuanced regulation. In our work, we observed such an increased Wnt activity only in HaCaT cells, but not in HDF. Of note, AHR's ability to modulate β-catenin levels extends beyond transcriptional control — acting as an E3 ubiquitin ligase, it can directly facilitate β-catenin degradation in a ligand-dependent manner, requiring molecules such as tryptophan or glucosinolates [43]. In fibroblasts, AHR may enhance, support, or maintain the Wnt/β-catenin pathway, whereas in keratinocytes it may restrain its expression. This may be due to the different role of the receptor in these cell types. Therefore, the mechanistic relationship between AHR and Wnt/β-catenin signaling is cell- and context-dependent and apparently does not appear to result solely from classical AHR activation through ligands and transcriptional regulation, suggesting post-translational regulation, protein-protein interaction, or impact on the stabilization of other pathway regulators. Moreover, in our hands, HDF-KO cells exhibited decreased transcription of analyzed Wnt/β-catenin pathway genes following TGFβ treatment, whereas HDF cells showed increased expression. This finding may support the anti-fibrotic role of the feedback loop between AHR and TGFβ.

At the same time, our combination experiments with TGFβ addition on wild-type versus AHR-deficient HDF cells revealed that collagen production triggered by TGFβ needs the AHR to reach optimal levels in these fibroblasts.

TGFβ and AHR – reciprocal regulation

Fibrosis is a natural part of the healing process, but under pathological conditions such as SSc, it becomes a complex phenomenon that reduces patients' quality of life, often causing significant pain and increasing mortality rates. In SSc, the extent of skin involvement correlates with a worse prognosis [44]. Although the AHR has been investigated in fibrosis, findings remain inconclusive or even contradictory [18, 45–49]. The AHR is expressed in all skin cells [50] and is well known for its cell-specificity and manifold, sometimes contradictory roles in regulating cellular functions [51, 52]. The AHR-TGFβ relationship is complex, as fibroblasts lacking the AHR secrete higher levels of TGFβ [53], whereas AHR activation is associated with reduced fibrotic responses [2, 54]. However, some inconsistencies have also been reported, particularly in intestinal studies [49, 55]. TGFβ downregulates AHR transcription, while the AHR modulates TGFβ signaling through interactions with key regulatory proteins such as latent TGFβ-binding protein (LTBP1) and SMADs. This reciprocal regulation highlights the complexity of the AHR-TGFβ interplay in fibrotic processes [56, 57]. It has long been known that the outcome of AHR activity is cell specific, and our data underscore this again. In HDF and HaCaT cells, removal of AHR or activation of AHR via the high-affinity ligand FICZ had different outcomes, as did the addition of TGFβ.

TGFβ and AHR – crosstalk in HDF and HaCaT

Under standard monoculture conditions, treatment of skin fibroblasts with TGFβ typically induces an increase in type I collagen secretion [35]. Interestingly, the co-culture of HDF and HaCaT resulted in lower collagen (COL1A1) production upon TGFβ treatment. This finding strongly suggests crosstalk between these two main skin-cell types of the epidermis and dermis. This clearly may have an impact on cellular functions, such as collagen production, cytokine secretion, and gene expression, as well as regulatory mechanisms under fibrotic conditions, which is consistent with the literature [4, 36].

Our results also confirmed that loss of the AHR in HDF abolished MMP1 secretion (Fig. 4), consistent with previous studies [7, 18], which demonstrated that AHR is an indispensable regulator of matrix degradation. Given that excessive collagen accumulation due to impaired MMP1 activity is a hallmark of fibrosis, our findings highlight the potential of the AHR as a therapeutic target for restoring ECM balance.

Cytokines

Moreover, in the co-culture of AHR-deficient cells, we observed disruptions and reduced signaling of proteins associated with rheumatoid arthritis, as well as cytokine interactions involving TNF and IL-17. An active AHR typically targets several genes, either directly or via a cascade triggered by those direct targets. As expected, proteome analysis identified several pathways, with the KEGG database indicating mainly inflammation-related events. This is consistent with the known role of AHR in immune balance. This sensitivity can be clinically important, as many SSc patients have low levels of AHR expression in skin fibroblasts. We observed that AHR-KO cells in co-culture secreted neither monocyte chemoattractant protein-1 (MCP-1) nor IL-8, both of which typically may be produced by keratinocytes and fibroblasts [58]. MCP-1 plays a role in inducing cellular senescence in keratinocytes [59] and stimulates inflammatory cells to the skin [60], whereas IL-8 may promote keratinocyte migration, which is important for wound healing [61]. While more research is needed, these data underscore the importance of the AHR in fibroblasts and their fibrogenic capacity.

Migration

A study using mouse skin tissue explants demonstrated that the lack of AHR accelerated skin re-epithelialization by enhancing keratinocyte migration, without affecting cell proliferation or the recruitment of inflammatory cells [62]. In contrast to this, we observed that AHR-deficient HaCaT cells were strongly impaired in closing the scratch gap within two days, and neither TGFβ nor FICZ addition helped. HaCaT cells were able to close the scratch gap, but HDF cells performed even better and faster (while chemical inhibition of AHR-signaling slowed this down). In both cell-types, the addition of FICZ and/or TGFβ accelerated this process. Note, however, that such scratch assay does not allow for differentiation between cell migration and proliferation. Additional functional tests, such as proliferation markers or time-lapse imaging, will be necessary to determine the exact mechanisms of this effect.

Immune cells

The proteome profiling data showed that immune-related proteins were modulated in the co-cultures. Keratinocytes are immune cells in the sense that they produce cytokines in response to injury, several of which were identified in the co-culture proteome profile (e.g. CXCL8 (IL8), MCP-1 (a monocyte attractant), or CCL5 (RANTES) - a T cell/eosinophil attractant. SSc is an autoimmune disease, thus immunological events are important. Due to the exploratory and rather qualitative nature of the proteome profiling, these observations should be interpreted as hypothesis generating, but are biologically consistent with the known function of keratinocytes and fibroblasts. More research is warranted on the role of the AHR in this context, i.e., the highly dynamic interactions between fibroblasts, keratinocytes, and immune cells. Therefore, future studies should incorporate immune components and address their involvement [63].

Conclusion

In conclusion, AHR-signaling is part of a three-directional crosstalk between AHR-TGFβ-Wnt/β-catenin in human dermal fibroblasts. This crosstalk was weaker or did not exist in HaCaT cells. Nonetheless, our co-cultivation experiments demonstrate a contribution of HaCaT cells to the fibrogenic process.

Our findings underscore the multifaceted, context-dependent nature of the AHR in fibrosis and skin homeostasis, highlighting distinct roles in fibroblasts and keratinocytes. Specifically, in fibroblasts, AHR appears crucial for balancing ECM turnover, whereas in keratinocytes, its role may be more structural. Consequently, rather than posing a simple "on/off" question regarding AHR, these results suggest that any therapeutic strategy may lie in the fine-tuning of AHR activity at the level of specific cell types—a challenging proposition, given the complexity of fibrotic processes and the diverse roles the AHR plays in different cellular environments.

For future studies, more refined co-culture models, including the incorporation of immune and endothelial cells, are important to examine the cross-talk between fibroblasts and keratinocytes. Such multifaceted models will provide a more comprehensive view of how AHR modulation might be therapeutically leveraged in systemic sclerosis and related fibrotic conditions.

Abbreviations

AHR aryl hydrocarbon receptor; ECM extracellular matrix; FICZ 6-formylindolo [3, 2-*b*] carbazole; HDF human dermal fibroblasts; KO knock-out; MMP1 matrix metalloproteinase 1; SSc Systemic sclerosis; TGFβ transforming growth factor beta; WT wild-type;

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Author Contributions

Conceptualization – A.W., C.E.; methodology – A.W., A. E-M, D.H.; formal analysis- B.S, A.W.; investigation- A. E-M, D.H, G.F., I.H, A.W.; generation of CRISPR-modified cell lines – A.R.; writing – original draft preparation - A.W., C.E.; writing—review and editing, A.G-P, T.H-S., C.E.

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Statement of Ethics

The authors have no ethical conflicts to disclose. This study did not involve human participants or experiments on animals.

Disclosure Statement

The authors have no conflicts of interest to declare.

Declaration of generative AI and AI-assisted technologies in the writing process
During the preparation of this work the authors used Grammarly and ChatGPT in order to improve the readability and language of the manuscript.

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