Supplementtary Material

Cellular Physiology and Biochemistry

Evidence For a Fibrogenic Interaction Between the Aryl Hydrocarbon Receptor and the Wnt/β-Catenin Pathways in Human Keratinocytes and Fibroblasts ; DOI: 10.33594/000000846

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1. Correlation in gene expression

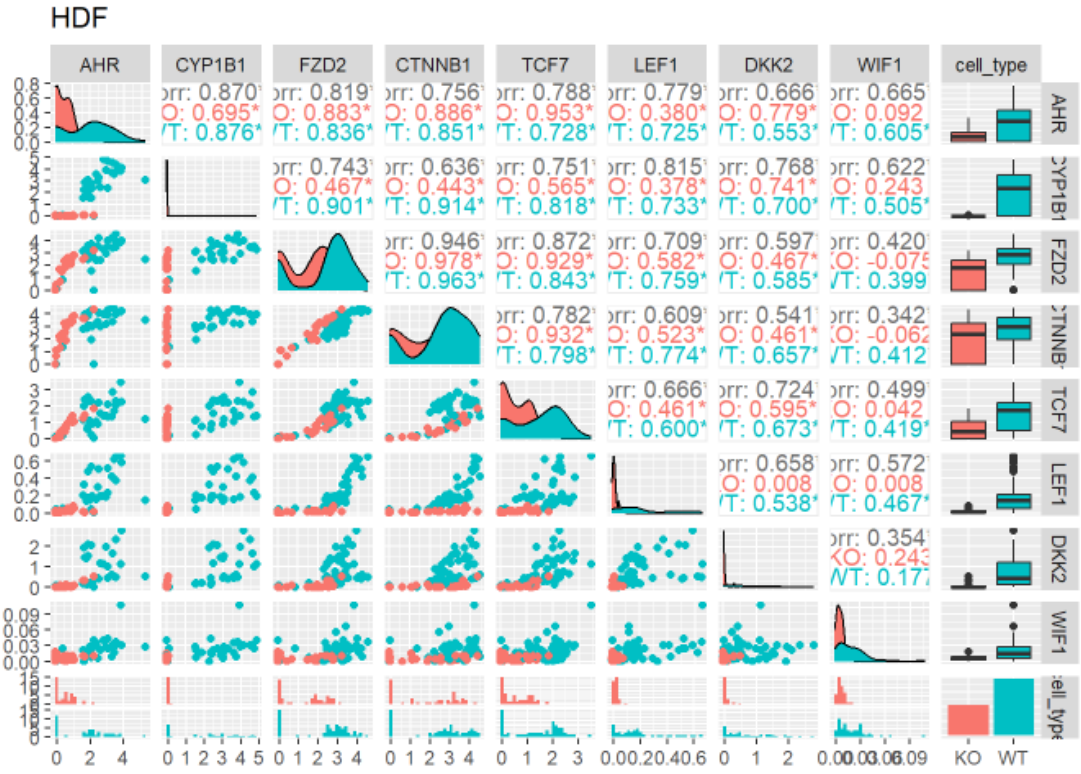

Figure S 1 Correlation of gene expression in wild-type (WT, blue) and AHR-knockout (KO, red) human dermal fibroblasts (HDF). The scatterplot matrix displays pairwise correlations between analyzed genes involved in AHR and Wnt/β-catenin signaling. The upper triangle shows correlation coefficients (corr) calculated for all samples (T), WT (blue), and KO (red). Density plots along the diagonal represent the distribution of expression levels for each gene. Boxplots on the right compare gene expression between WT and KO cells.

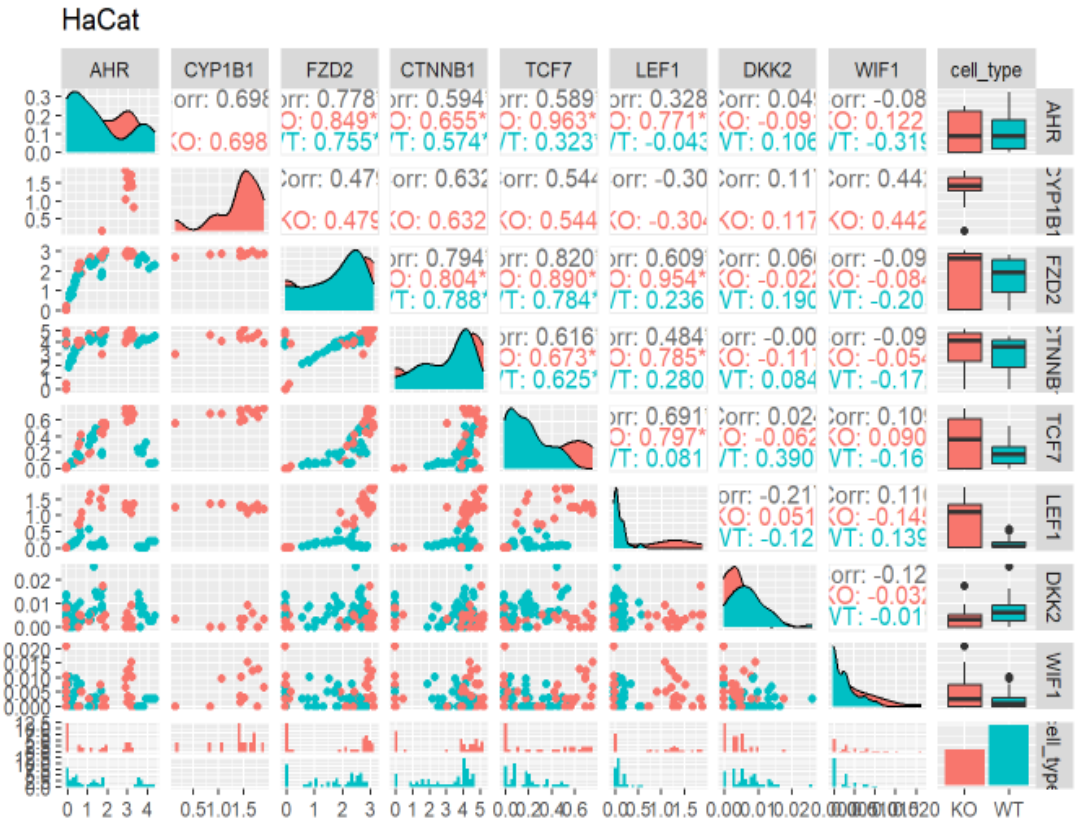

Figure S 2 Correlation of gene expression in wild-type (WT, blue) and AHR-knockout (KO, red) human keratinocytes (HaCat). The scatterplot matrix displays pairwise correlations between analyzed genes involved in AHR and Wnt/β-catenin signaling. The upper triangle shows correlation coefficients (corr) calculated for all samples (T), WT (blue), and KO (red). Density plots along the diagonal represent the distribution of expression levels for each gene. Boxplots on the right compare gene expression between WT and KO cells.

2. Methylation Analysis

a. DNA extraction and bisulfite conversion

The human dermal fibroblasts (HDF) cell line were maintained in DMEM medium, supplemented with 10% fetal bovine serum, treated 10 ng/ml and 25 ng/ml TGFβ, and untreated HDF after 24 and 48 hours. DNA was extracted from HDF using Xpure™Cell&Tissue micro, according to the manufacturer's protocol (A&A Biotechnology, Gdansk, Poland, Cat. No. 090-50). The quantity and quality of isolated DNA were measured with spectrophotometer (Denovix Inc., Ellington, USA). Sodium bisulfite conversion of genomic DNA was performed on 400 ng DNA using a CiTi Converter DNA Methylation Kit, according to the manufacturer's protocol (A&A Biotechnology, Gdansk, Poland, Cat. No. 027-250).

b. Human *WIF1, LEF1* and *DKK2* promoter prediction and methylation-specific PCR (MSP)

We selected an approximately 360 kb DNA sequence upstream of the *WIF1, LEF1, DKK2* transcription start site as the promoter region. *DKK2* was also included into the analysis because of its relatively low level of expression. The promoter sequence was predicted using the UCSC Genome Browser on Human (GRCh38/hg38) (https://genome.ucsc.edu/). The specific primer sets for the PCR reactions were designed using MethPrimer 2.0 software (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). *WIF1, LEF1, DKK2* gene methylation was determined using primer pairs used for methylation analysis and PCR size products are shown in ***Table 1 S***. MSP was performed using CiTi Converter MSP PCR Kit (A&A Biotechnology, Gdansk, Poland, Cat. No. 1080-100). As a positive control for methylation DNA that has been enzymatically methylated at all cytosines within a CpG dinucleotide was used (Zymo Research, Cat. No. D5015). A water blank (no template DNA) was also included as a negative PCR control. The products were separated in 2% agarose gels using a DNA Marker 1 (A&A Biotechnology, Gdansk, Poland, Cat. No. 3000-500) marker set.

Table 1 S. Methyl-specific polymerase chain reaction primer sets, extracted from the MethPrimer 2.0.

Gene name	Primer unmethylated sequence, 5'→3	Product size	Primer methylated sequence, 5'→3	Product size
WIF1 (promoter 2)	F: GAAAATTTTTTGTGTTGTATTTATGT	133	F: GGAAAATTTTTCGTGTCGTATTTAC	132
	R: AAACTCCTTAACACCCAAACCA		R: ACTCCTTAACACCCAAACCG	
LEF1 (promoter 1)	F: GTTTTGTTAAAGTAAAGAGTTGTGT	275	F: GTTTTGTTAAATAAAGAGTTGCGT	277
	R: CAACCAAAAAAAACTAAAAACAAA		R: AACAACCAAAAAAAACTAAAACGA	
DKK2 (promoter 2)	F: TATGTTTTAGGATAGAAATTAGTGG	138	F: TTACGTTTTAGGATAGAAATTAGCG	141
	R: ATTTTCTTACACCTTAAATCACACC		R: AAATTTTCTTACACCTTAAATCACG	

[i] F, forward'; R, reverse; WIF1, WNT inhibitory factor 1; LEF1, lymphoid enhancer binding factor 1; DKK2, dickkopf WNT signaling pathway inhibitor 2

Methylation-specific PCR analysis revealed the presence of methylated bands in promoter for WIF1 (Figure S3 C) and LEF1 (Figure S4 C), and both methylated and unmethylated bands for DKK2 (Figure S5 C).

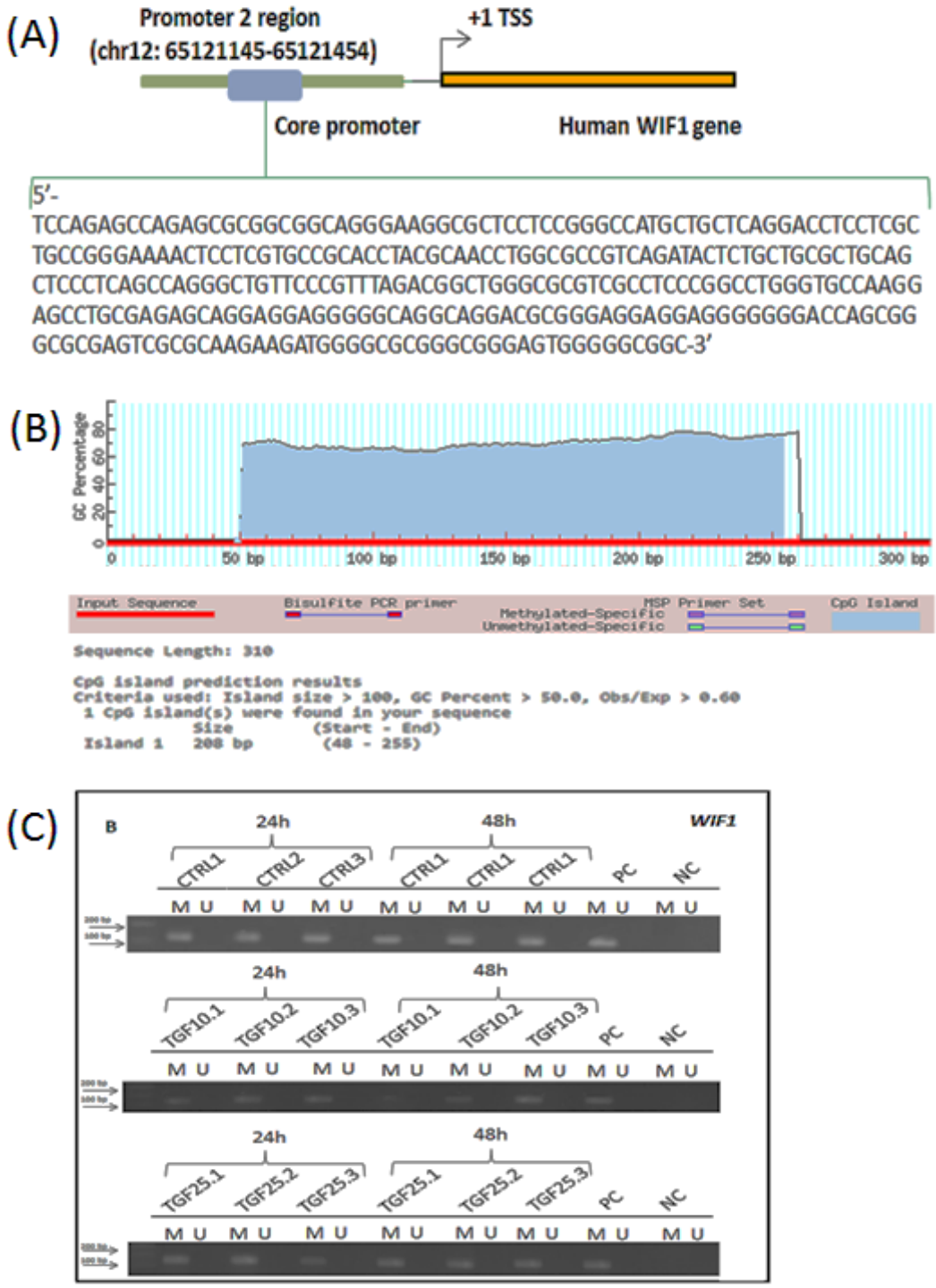

Figure S 3 ***(A)*** *Prediction and methylation detection of CpG island in WIF1 gene promoter.* ***(B)*** *Verification of promoter activity of WIF1 gene.* ***(C)*** *Methylation status of the WIF1 promoter region shown in triplicates from HDF line treated 10 ng/ml and 25 ng/ml TGFβ, and untreated HDF after 24 and 48 hours. M – methylated; U-unmethylated; NC -negative control, PC – positive control; CTRL- samples without treatment; TFG10 and TGF25 – samples treated with 10 ng/ml or 25 ng/ml of TGFβ*

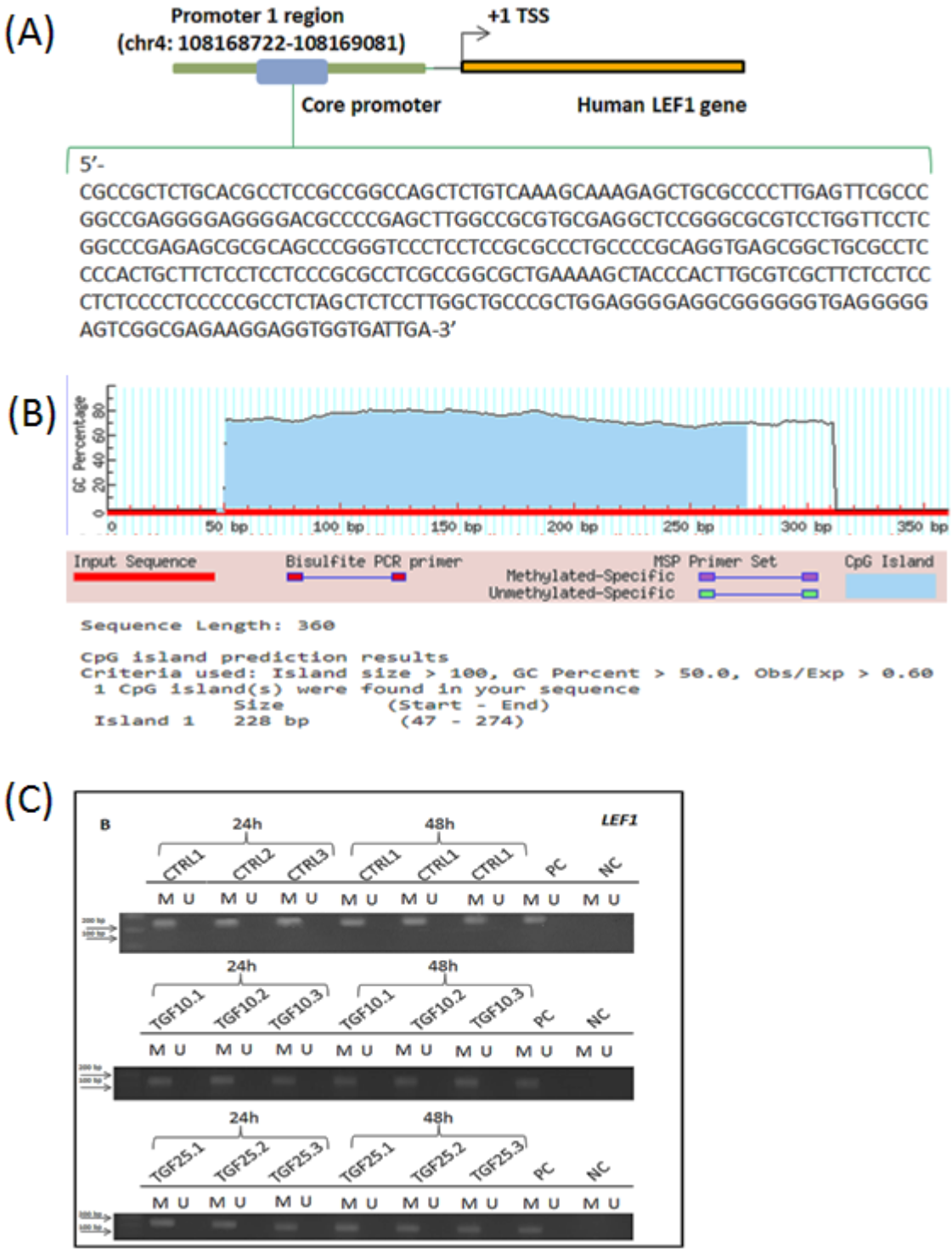

Figure S 4 ***(A)*** *Prediction and methylation detection of CpG island in LEF1 gene promoter.* ***(B)*** *Verification of promoter activity of LEF1 gene.* ***(C)*** *Methylation status of the LEF1 promoter region shown in triplicates from HDF line treated 10 ng/ml and 25 ng/ml TGFβ, and untreated HDF after 24 and 48 hours. M – methylated; U-unmethylated; NC -negative control, PC – positive control; CTRL- samples without treatment; TFG10 and TGF25 – samples treated with 10 ng/ml or 25 ng/ml of TGFβ*

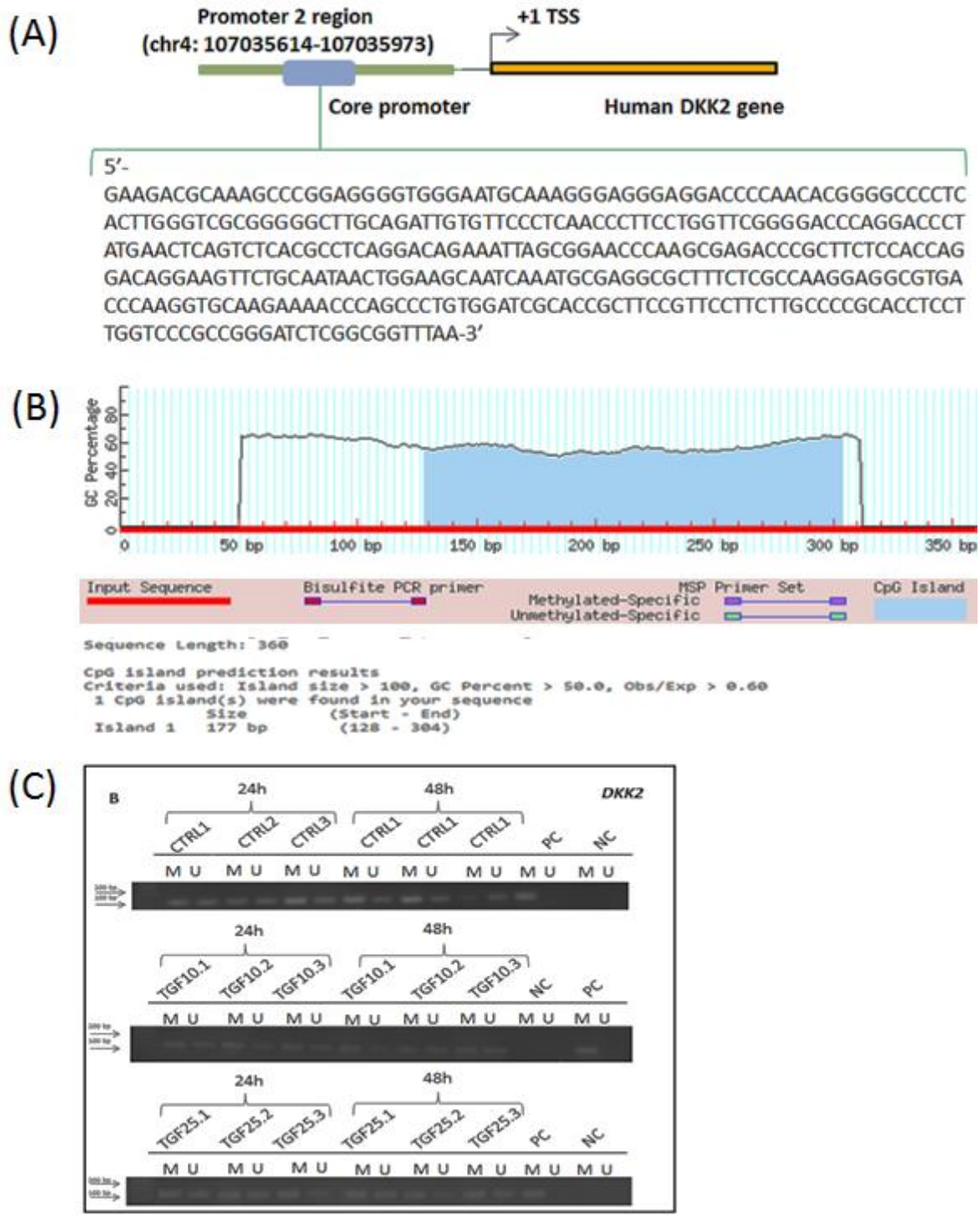

Figure S 5 ***(A)*** *Prediction and methylation detection of CpG island in DKK2 gene promoter.* ***(B)*** *Verification of promoter activity of DKK2 gene.* ***(C)*** *Methylation status of the DKK2 promoter region shown in triplicates from HDF line treated 10 ng/ml and 25 ng/ml TGFβ, and untreated HDF after 24 and 48 hours. M – methylated; U-unmethylated; NC -negative control, PC – positive control; CTRL- samples without treatment; TFG10 and TGF25 – samples treated with 10 ng/ml or 25 ng/ml of TGFβ*

3. Proteome profiler

Table S2 Comparative Analysis of Secreted Protein Expression in WT's Cells (Co-culture Model of HDF wt and HaCat wt) and KO's Cells (Co-culture Model of HDF KO and HaCat KO) Using Proteome Profiler

Uniprot ID	Secreted protein	Co-culture model type		Change
		wt/wt	KO/KO	
P05121	Serpin E1	+++	+++	No Change
O94907	Dkk-1	+++	+++	No Change
P80188	Lipocalin-2	++	++	No Change
P13501	RANTES (CCL5)	+	++	Increase
P17936	IGFBP-3	+	++	Increase
P09341	GROα (CXCL1)	++	-	Loss
P42830	ENA-78 (CXCL5)	++	-	Loss
P05231	IL-6	++	-	Loss
P10145	IL-8 (CXCL8)	+++	-	Loss
P13500	MCP-1	+++	-	Loss
P26022	Pentraxin 3 (PTX3)	+	-	Loss
P07996	Thrombospondin-1 (THBS1)	+	-	Loss
P04141	GM-CSF	++	-	Loss
P15692	VEGF (VEGFA)	+	-	Loss
Q15848	Adiponectin	-	+++	Increase
P10451	Osteopontin (SPP1)	-	+	Increase
P04278	SHBG	-	++	Increase
P02647	Apolipoprotein A-I	-	++	Increase
Q10586	Vitamin D BP	-	+++	Increase
P03950	Angiogenin	-	+++	Increase
P16284	CD31	-	+	Increase
P02776	PF4	-	+++	Increase
P02786	TfR	-	++	Increase
P01031	Complement Component C5/C5a	-	++	Increase
P02753	RBP-4	-	+++	Increase
P14780	MMP-9	-	++	Increase

*Signal intensity was assessed based on detection levels: **"-" (no signal), "+" (weak), "++" (moderate), and "+++" (strong).** The table presents the **Uniprot ID**, **secreted protein names**, and their corresponding expression levels in **WT's cells (co-culture of HDF wt and HaCat wt)** and **KO's cells (co-culture of HDF KO and HaCat KO)**. The final column indicates the observed **changes in protein expression** between the two conditions, categorized as **"No Change," "Increase," or "Loss."***

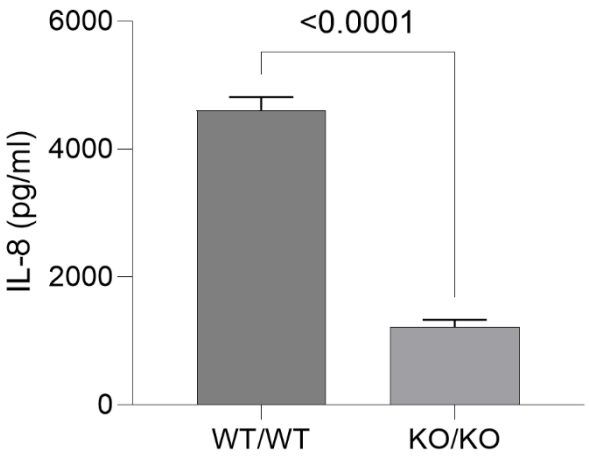

Figure S 6 Concentration of IL-8 in supernatants in WT's Cells (Co-culture Model of HDF wt and HaCat wt) and KO's Cells (Co-culture Model of HDF KO and HaCat KO) analyzed by ELISA test (KE00006, Proteintech). HDF cells were seeded in the lower chamber, and HaCaT cells were cultured on an insert. Unpaired t test. Barplot. Mean± SD. $p \leq 0.05$