Research letter

Isotretinoin is indirectly effective in sebocytes

DOI: 10.1111/bjd.18562

Dear Editor, In a recently published research letter, Burney *et al.* demonstrated that isotretinoin (13-*cis*-retinoic acid, 13CRA) induced acnegenic changes in cultured human SEB-1 sebocytes.[1] However, Melnik *et al.*[2] suggested that the observed changes may largely be influenced by the Simian virus 40 (SV40) immortalization of the used cell line, which may inhibit the p53 pathway and, together with 13CRA, lead to changes such as downregulation of the forkhead box O (FOXO) pathway. In contrast, they found that the FOXO pathway was upregulated in samples from patients receiving 13CRA treatment for 6 weeks.[2] Importantly, the prominent differences, that 13CRA treatment arrests the cell cycle and induces apoptosis in SEB-1 sebocytes while in SZ95 sebocytes (a cell line also immortalized with the SV-40 large T antigen) 13CRA treatment does not affect programmed cell death,[3,4] suggest that SV-40 immortalization is not a fate-determining factor in these cell lines.

Looking beyond these limitations, which arise from matching findings from a cell line with data from *in vivo* samples obtained at a given time point, the most challenging question regarding the effects of 13CRA is whether it is, indeed, the molecule responsible for the *in vivo* and *in vitro* observed changes. Besides having a weak binding affinity to its target receptors, RARs (retinoic acid receptors) and RXRs (retinoid X receptors), and a poor potential for direct transcriptional activation, 13CRA rapidly isomerizes to all-*trans* RA (ATRA) and other RA isomers. Therefore, it is generally assumed that 13CRA exerts its biological effect by serving as a precursor for ATRA and/or 9-*cis*-retinoic acid isomers, which can efficiently activate RARs and RXRs or even both in combination. This is further supported by the findings that 13CRA treatment results in low intracellular 13CRA and high intracellular ATRA levels in sebocytes.[5]

To assess the direct changes that 13CRA could exert in sebocytes we treated SZ95 sebocytes[6] with 13CRA for 6 h and 24 h, and collected samples for RNA-Seq analysis to reveal genome-wide changes. While the 6-h time point – when conversion of 13CRA to its metabolites is still limited – allowed us to identify direct changes, the 24-h time point, when 13CRA is already converted,[5] revealed the long-term changes at the level of gene expression. Our RNA-Seq analysis confirmed that of the 361 genes showing significantly changed expression levels at 6 h, only 45 remained significantly upregulated at 24 h in response to 13CRA (Fig. 1a). Further, the majority of these 45 genes had a decreased fold change at 24 h when compared with the values detected in the 6-h samples (full data available on request). These findings confirm that the effects of 13CRA are rather temporary on gene expression level in sebocytes.

To link the detected changes to possible cellular functions, we clustered the differentially expressed genes at 6 h using the PANTHER Classification System (pantherdb.org). The clustering revealed that gene expression changes were mostly related to an altered growth factor and to other differentiation pathways such as Wnt (the key pathway in sebocyte differentiation) but not to lipid metabolism. When clustering genes from the 6-h time point that remained significantly altered at 24 h, results confirmed a central role for the phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma gene (*PIK3CG*, also known as PI3K-gamma) and its related pathways (p53, VEGF, Ras, hypoxia, IGF, EGF, FGF, PDGF, apoptosis and angiogenesis signalling); which was shown to be upregulated also in the non-SV-40 immortalized U937 myeloid cells on ATRA treatment.[7] Moreover, 13CRA activated the PIK3CG/Akt/FOXO pathway not only in SZ95 sebocytes *in vitro* but also in sebaceous glands of 13CRA-treated patients with acne.[8] Importantly, when comparing the lists of those genes regulated by 13CRA with a list of the immediately responding target genes of ATRA (3 h after ATRA treatment) in SZ95 sebocytes, we found a limited overlap (Fig. 1b). Such a weak signature of a possible ATRA-related effect in the 13CRA-induced gene expression profile and the related functional clusters (Fig. 1c) suggests that mechanisms involving alternative 13CRA metabolites may also have to be taken into account to explain the therapeutic effects of 13CRA treatment.

In conclusion, our RNA-Seq analysis supports the hypothesis that 13CRA is, rather than the direct effective agent, a precursor for alternative bioactive retinoids. To develop novel retinoids with better efficacy and fewer adverse effects, it is essential to understand in more detail the overall molecular mechanisms of RA metabolism, such as dehydrogenation and isomerization, which are distinct from the conversion of 13CRA to ATRA (Fig. 1d).

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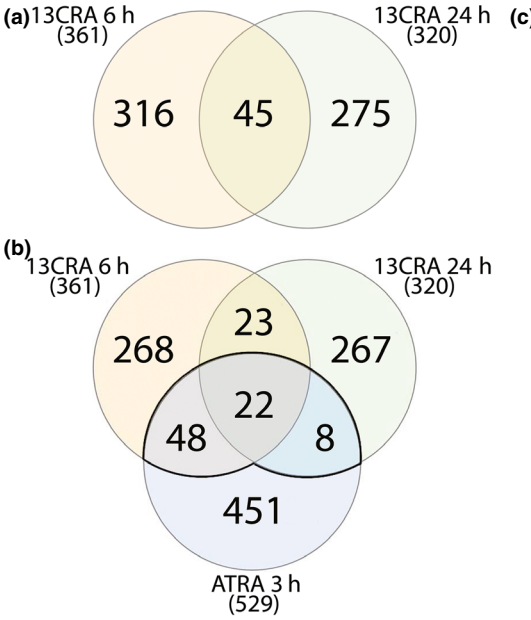

(c) Pathway	Gene name
5HT1 type receptor signalling	GNAO1
Angiogenesis	PIK3CG, PLA2G4D, PTPRB, PDGFRA, AXIN2, WNT7B
Apoptosis signalling	PIK3CG, BCL2A1, TNFSF10
Axon guidance	SLIT1, PIK3CG
B cell activation	CD22, PIK3CG, BLK
Blood coagulation	PROC, THBD
Cadherin signalling	WNT7B, CDH5
EGF receptor signalling	PIK3CG
Endogenous cannabinoid signalling	GNAO1
GnRH receptor	PTGER2, NPPC, GNAO1
Inflammation mediated signalling	PIK3CG, ITGA4, CAMK2A, GNAO1
Insulin/IGF	INSRR, PIK3CG, INSRR
Integrin signalling	PIK3CG, ITGA4, LAMB4
Glutamate receptor	CAMK2A, GRIA4, GNAO1
Oxidative stress response	TXNDC2, DUSP27
PDGF signalling	PIK3CG, NINL, ELF3, PDGFRA
Ras	PIK3CG
T cell activation	PIK3CG
TGF-beta signalling	BMP5
VEGF signalling	PIK3CG
Vitamin D	CYP24A1
Wnt signalling	AXIN2, WNT7B, CDH5
p53	PIK3CG

13CRA 6 h **13CRA 6 h/13CRA 24 h/ATRA 3 h**

Fig 1. (a, b) Venn diagrams showing the numbers of significantly regulated genes from the RNA-Seq analysis of SZ95 sebocytes when treated with: 1 μmol L^{-1} 13-*cis*-retinoic acid (13CRA) for 6 h and 24 h (a), and with 1 μmol L^{-1} 13CRA for 6 h and 24 h and 1 μmol L^{-1} all-*trans* RA (ATRA) for 3 h (b), compared with untreated sebocytes. (c) Pathway analysis of the differentially expressed genes. In black are the clusters and related genes that were differentially expressed in sebocytes 6 h after 13CRA treatment, while in red are those that were differentially expressed in all of the examined conditions. (d) Metabolic pathways starting from 13CRA to known RA isomers and to dihydroretinoids, which may confer additional, still unknown, therapeutic effects. ATDHRA, all-*trans*-13,14-dihydroretinoic acid; DCRA, di-*cis*-RA; 9CDHRA, 9-*cis*-13,14-dihydroretinoic acid; 5HT, 5-hydroxytryptamine; EGF, epidermal growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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Funding sources: Hungarian National Research Fund (NN117020), Marie Skłodowska-Curie actions (627547 RNAsebgland FP7-PEOPLE-2013-IEF) to D.T., and GINOP-2·3·2-2016-00005 to D.T., D.K. and K.H., cofinanced by the European Union and the European Regional Development Fund. D.T. is a recipient of the János Bolyai research scholarship of the Hungarian Academy of Sciences. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of interest: C.C.Z. owns an international patent on the SZ95 sebaceous gland cell line (WO2000046353).