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Voriconazole exposure regulates distinct cell-cycle and terminal differentiation pathways in primary human keratinocytes

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Research letter

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Dear Editor, Voriconazole is a second-generation triazole antifungal used routinely in the care of solid-organ and stem-cell transplant recipients to both prevent and treat invasive fungal infections.[1] Voriconazole causes photosensitivity,[2] and has been associated with a dose-dependent increased risk of developing cutaneous squamous cell carcinoma (cSCC).[3] Possible mechanisms suggested include direct phototoxicity by voriconazole or one of its metabolites,[4] oxidative stress pathway activation,[5] an indirect retinoid effect or DNA damage repair inhibition.[6] However, the exact role of voriconazole in cSCC carcinogenesis remains poorly understood.

In this study, we sought to identify molecular mechanisms of voriconazole-associated carcinogenesis in cSCC. We hypothesized that voriconazole or voriconazole N-oxide (VNO), it primary drug metabolite, might regulate key signalling pathways related to cSCC carcinogenesis. To investigate broadly these hypotheses, we used gene expression arrays of *in vitro* cultures of primary human keratinocytes (PHKs) and human cSCC B12 (SCCB12) cell lines exposed to either voriconazole or VNO (135 μmol L^{-1}) for 14 days (Appendix S1; see Supporting Information). The institutional review board at the University of California, San Francisco, approved this study.

Principal component analysis of array data demonstrated that drug exposure primarily regulated gene expression in PHKs (rather than SCCB12 cells) with voriconazole (rather than VNO) as the primary driver of variance (Fig. 1a, b). Differential gene expression analysis (fold change ≥ 1·5; false discovery rate $Q < 0{\cdot}05$) identified 318 voriconazole-regulated genes (Table S1; see Supporting Information) and 36 VNO-regulated genes (Table S2; see Supporting Information) in PHKs. In SCCB12 cells no gene targets were significantly regulated by drug exposure. Supervised hierarchical clustering was used to identify two primary clusters of gene targets regulated by drug exposure in PHKs that were also differentially expressed in cSCC (Fig. 1c). Cluster 1 consisted of 197 unique genes upregulated in voriconazole-exposed PHKs (Table S3; see Supporting Information), including cell-cycle pathway regulators such as *CDC2*, *DLGAP5*, *CDKN3* and *NDC80*. Cluster 2 consisted of 76 unique genes downregulated in voriconazole-exposed PHKs (Table S4; see Supporting Information), including markers of keratinocyte terminal differentiation such as *SPRR3*, *LCE3E* and *IVL*.

To identify biological themes, we performed functional enrichment and gene-set enrichment analysis. Voriconazole upregulated genes related to cell division, including chromosome condensation, DNA replication, spindle organization and cell-cycle checkpoint control (Table S5; see Supporting Information), as well as a variety of cancer-related pathways, including oncogenic Ras signalling and its downstream target forkhead box protein M1 [FOXM1; Tables S6 and S7 (see Supporting Information)]. Voriconazole downregulated genes related to terminal epithelial differentiation and protease inhibitor activity (Table S8; see Supporting Information), in addition to pathways related to tumour gene silencing, de-differentiation, metastasis, and oncogenic Ras and nuclear factor kappa B signalling (Tables S9 and S10; see Supporting Information). Differentially expressed genes with important functional significance were further validated using quantitative polymerase chain reaction (PCR), demonstrating upregulation of *FOXM1* and its downstream targets *CEP55*, *PLK1* and *AURKB* (Fig. 1d), and downregulation of *IVL*, *LCE3E*, *S100A12* and *SPRR3* (Fig. 1e).

Next, we used three-dimensional organotypic cultures (OTCs) of PHKs exposed to fluconazole or voriconazole (270 μmol L^{-1}) to investigate whether voriconazole exposure affected the structure and development of human skin. Fluconazole, another triazole used for antifungal prophylaxis,[7] was utilized to assess for drug class effect. Voriconazole exposure inhibited terminal differentiation, resulting in poor formation of the granular and cornified epithelial layers, but there were no significant differences between fluconazole and untreated samples (Fig. 2a). We found a similar, but reduced, effect at the lower voriconazole dose (135 μmol L^{-1}), indicating a dose-dependent relationship (Fig. S1a; see Supporting Information). We also found reduced protein expression of terminal epithelial markers in voriconazole-treated PHKs (Fig. 2b). *FOXM1* expression was low across all conditions [positive control; Fig. S1b (see Supporting Information)], although we found basal layer cell proliferation across all conditions indicated by positive Ki67 staining (Fig. 2b). Gene expression of key markers were further validated with quantitative PCR (Fig. 2c, d).

These data provide evidence that voriconazole regulates distinct cell-cycle and terminal differentiation pathways in PHKs, offering novel insights into the poorly understood mechanism of voriconazole-associated carcinogenesis. Voriconazole exposure inhibited expression of terminal epithelial differentiation markers, most comprising the epithelial differentiation complex (EDC), a set of genes coexpressed in outer epithelial layers and clustered on chromosomal region 1q21.[8] EDC genes

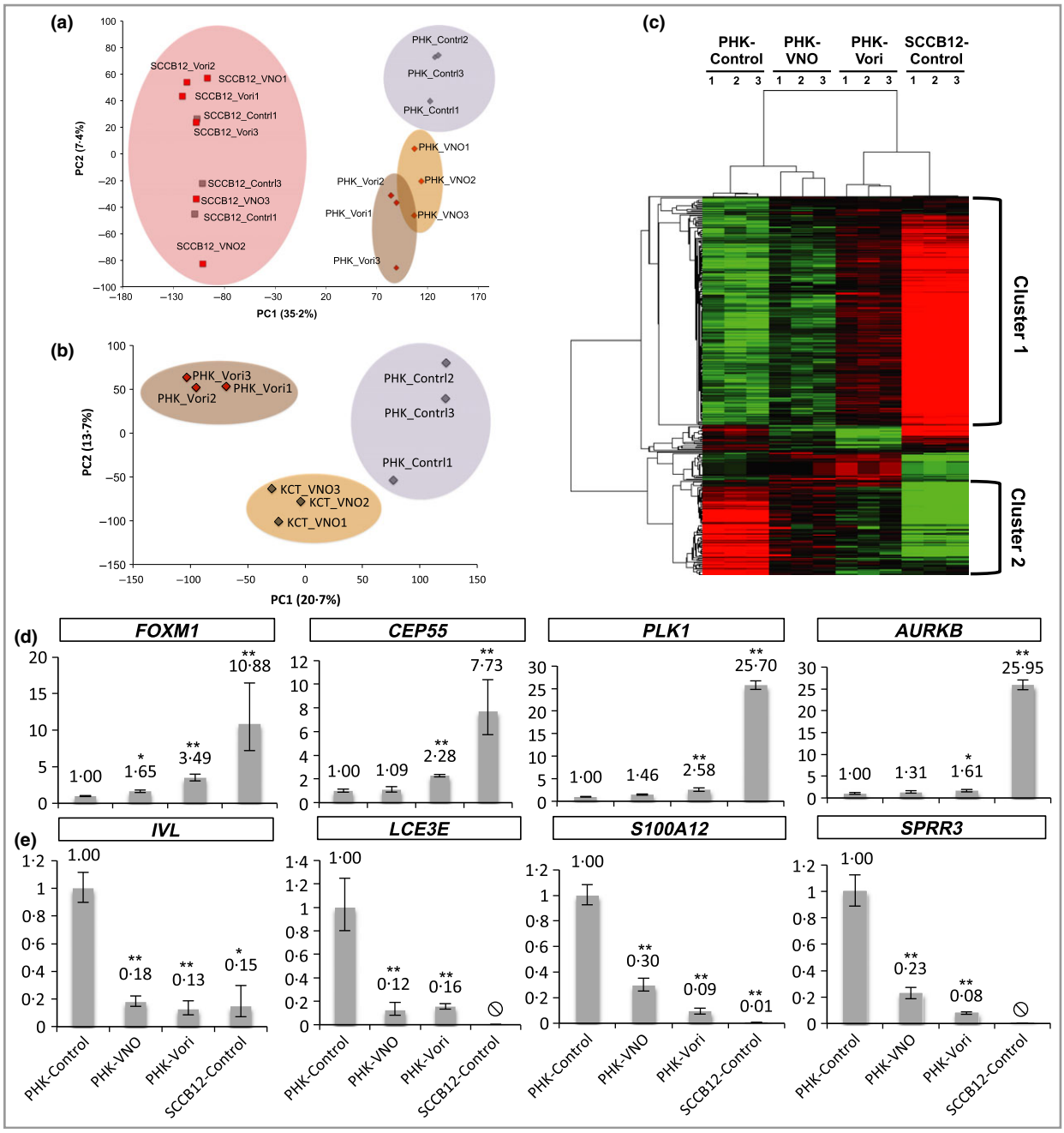

Fig 1. Principal component analysis (PCA), supervised hierarchic clustering analysis of gene expression data and quantitative polymerase chain reaction validation of gene expression results. (a) PCA of entire normalized dataset showing PC1 (*x*-axis) and PC2 (*y*-axis). (b) PCA of restricted dataset limited to primary human keratinocyte (PHK) conditions showing PC1 (*x*-axis) and PC2 (*y*-axis). (c) Supervised hierarchical clustering and heat map of differentially expressed gene between untreated (PHK-Control) and voriconazole N-oxide-treated (VNO; PHK-VNO) or voriconazole-treated (PHK-Vori) PHKs (fold change ≥ 1·5, false discovery rate < 0·05) compared with untreated cutaneous squamous cell carcinoma (SCCB12) cell lines (SCC-Control). Expression values were log2-adjusted and median-normalized. Clusters 1 and 2 are identified to the right of the heat map. (d) Average fold expression change of voriconazole and VNO-treated PHKs (PHK-Vori and PHK-VNO, respectively) and untreated SCCB12 (SCCB12-Control) cell lines relative to untreated PHKs (PHK-Control) for selected cluster 1 genes (*FOXM1*, *CEP55*, *PLK1* and *AURKB*). (e) Average fold expression change of voriconazole and VNO-treated PHKs (PHK-Vori and PHK-VNO, respectively) and untreated SCCB12 (SCCB12-Control) cell lines relative to untreated PHKs (PHK-Control) for selected cluster 2 genes (*IVL*, *LCE3E*, *S100A12* and *SPRR3*). Two-sided t-tests were performed to assess statistical significance relative to untreated PHKs (PHK-Control). *P < 0·05; **P < 0·01. The circle with diagonal line indicates undetectable gene expression.

are typically overexpressed in early cSCC lesions, likely indicating hyperplasia, but are eventually downregulated following squamous cell dedifferentiation after malignant transformation.[9] In OTCs, voriconazole inhibited terminal differentiation, resulting in poor formation of the granular and corneal epithelial layers. Functional stratum corneum is

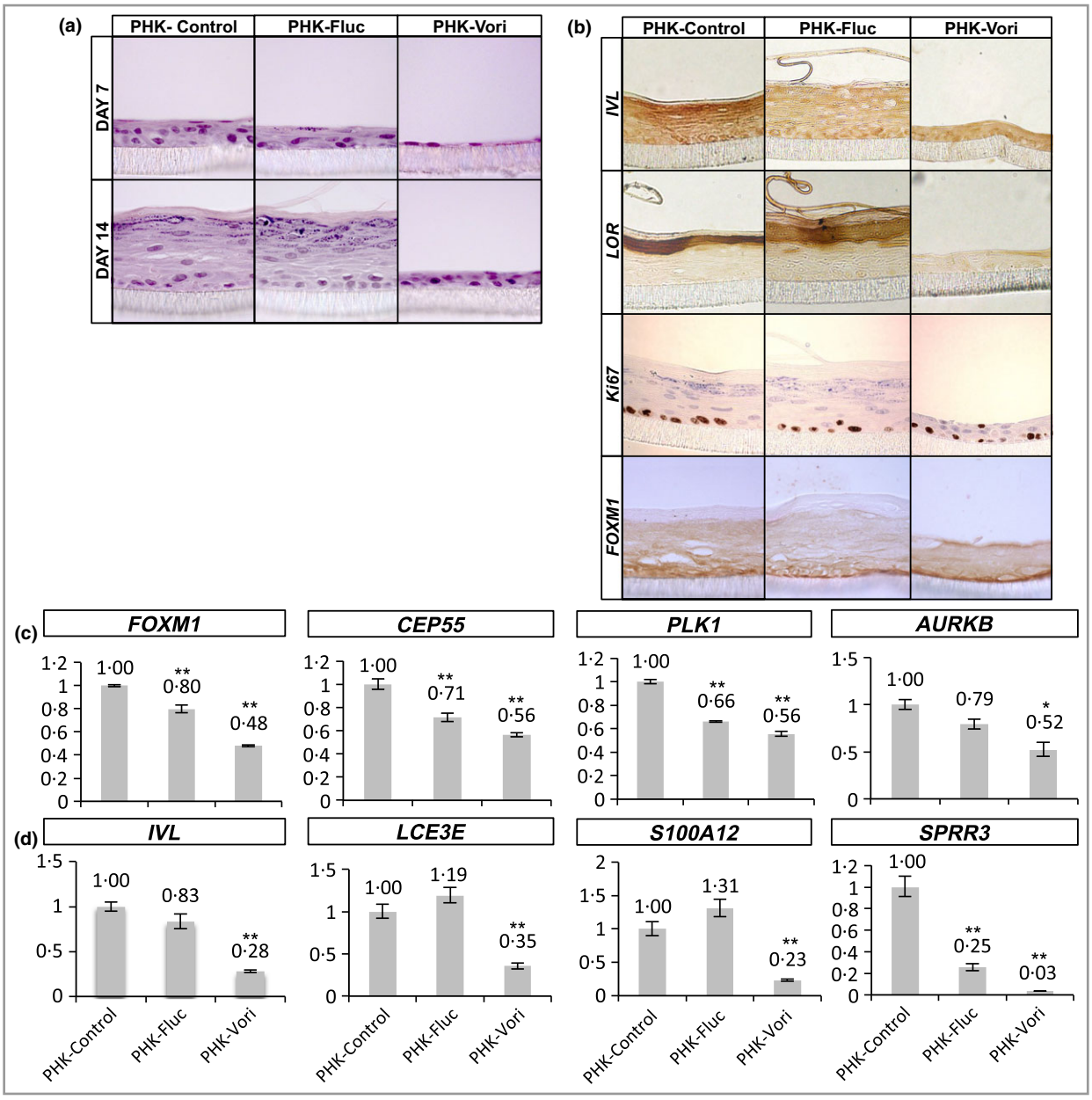

Fig 2. Haematoxylin and eosin and immunhistochemical staining of untreated and drug-treated primary human keratinocytes (PHKs) in organotypic cultures with quantitative polymerase chain reaction validation of gene expression. (a) Haematoxylin and eosin-stained sections of untreated (PHK-Control), fluconazole-treated (PHK-Fluc) and voriconazole-treated (PHK-Vori) PHKs grown as organotypic cultures (OTCs) isolated on days 7 and 14. (b) Immunohistochemically stained sections of untreated (PHK-Control), fluconazole-treated (PHK-Fluc) and voriconazole-treated (PHK-Vori) PHKs grown as OTCs isolated on day 14. Samples were stained with antibodies against involucrin (IVL), loricrin (LOR), fillaggrin (FLG) and forkhead box protein M1 (FOXM1), and visualized using an avidin–biotin complex peroxidase system. (c) Average fold expression change of fluconazole and voriconazole-treated PHKs in OTC (PHK-Fluc and PHK-Vori, respectively) relative to untreated PHKs in OTC (PHK-Control) for selected cluster 1 genes (*FOXM1*, *CEP55*, *PLK1* and *AURKB*). (d) Average fold expression change of fluconazole and voriconazole-treated PHKs in OTC (PHK-Fluc and PHK-Vori, respectively) relative to untreated PHKs in OTC (PHK-Control) for selected cluster 2 genes (*IVL*, *LCE3E*, *S100A12* and *SPRR3*). Two-sided *t*-tests were performed to assess statistical significance relative to untreated PHKs (PHK-Control). *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

important for photoprotection owing to its physical barrier effect and its production of urocanic acid, which absorbs ultraviolet (UV) light;[10] it is thus possible that the impact of voriconazole on terminal differentiation might be synergistic with its UVA-sensitizing properties,[4] and contribute to chronic photosensitivity. However, further studies are needed to assess the impact of voriconazole exposure on terminal differentiation in the context of UV exposure.

Voriconazole exposure also regulated cell-cycle pathways, including the FOXM1 tumorigenesis pathway. FOXM1 is a key transcription factor expressed in proliferating cells and regulates distinct cell-cycle checkpoints, largely through its effect on numerous downstream targets such as CDC25B, cyclin B1, aurora kinase B, centromere protein F family genes and survivin.[11] FOXM1 overexpression has been linked to the majority of human malignancies,[12] including epithelial cancers such as cSCC.[13] In keratinocytes, FOXM1 overexpression has been shown to result in genomic instability potentiated by UVB exposure, likely representing a first hit in skin carcinogenesis.[14]

In conclusion, this study identifies that voriconazole regulates distinct cell-cycle and terminal differentiation pathways in normal keratinocytes. These effects appear to be unique to voriconazole exposure, rather than a general azole class effect, likely owing to unique structural differences between individual azole antifungals.[15] Future *in vitro* and *in vivo* studies are needed to further explore the relationship between voriconazole, terminal differentiation and cell-cycle regulation, particularly in the context of UV exposure. Pathway effects of other azoles used routinely in the care of lung transplant recipients, such as itraconazole or posaconazole, should also be investigated. Further characterization of voriconazole-associated mechanisms of carcinogenesis utilizing these findings may lead to a better understanding of the key signalling pathways controlling cSCC and offer novel insights into preventing skin cancer in patients receiving voriconazole.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig S1. Haematoxylin and eosin staining of untreated and drug-treated (low and high dose) primary human keratinocytes in organotypic cultures and forkhead box protein M1 positive-control staining of cutaneous squamous cell carcinoma (SCCB12) monolayer cultures.

Table S1. Average relative fold change of differentially expressed genes in voriconazole-treated vs. untreated primary human keratinocytes.

Table S2. Average relative fold change of differentially expressed genes in voriconazole N-oxide-treated vs. untreated primary human keratinocytes.

Table S3. Average relative fold change of cluster 1 gene targets in voriconazole N-oxide-treated primary human keratinocytes, voriconazole-treated primary human keratinocytes and untreated squamous cell carcinoma vs. untreated primary human keratinocytes.

Table S4. Average relative fold change of cluster 2 gene targets in voriconazole N-oxide-treated primary human

keratinocytes, voriconazole-treated primary human keratinocytes and untreated squamous cell carcinoma vs. untreated primary human keratinocytes.

Table S5. Database for Annotation, Visualization and Integrated Discovery functional enrichment analysis of cluster 1 gene targets.

Table S6. Gene set enrichment analysis of genes enriched in voriconazole-treated primary human keratinocytes relative to untreated primary human keratinocytes (upregulated by voriconazole) compared with the C1 (Gene Ontology) database.

Table S7. Gene set enrichment analysis of genes enriched in voriconazole-treated primary human keratinocytes relative to untreated primary human keratinocytes (upregulated by voriconazole) compared with the C2 (Curated) and C6 (Oncogenic Signatures) databases.

Table S8. Database for Annotation, Visualization and Integrated Discovery functional enrichment analysis of cluster 2 gene targets.

Table S9. Gene set enrichment analysis of genes enriched in untreated primary human keratinocytes relative to voriconazole-treated primary human keratinocytes (downregulated by voriconazole) compared with the C1 (Gene Ontology) database.

Table S10. Gene set enrichment analysis of genes enriched in untreated primary human keratinocytes relative to voriconazole-treated primary human keratinocytes (downregulated by Voriconazole) compared with the C2 (Curated) and C6 (Oncogenic Signatures) databases.

Appendix S1. Supplemental 'Materials and methods'.

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