Experimental Dermatology

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Targeting XIAP and EGFR as an Approach for Treating Melanoma

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Melanoma is one of the deadliest forms of cancer, accounting for the majority of skin cancer-related deaths due to its high invasiveness and tendency to metastasise. Early diagnosis and resection remain the best options for treating melanoma. Still, several new targeted therapy approaches, such as targeting BRAF and MEK, have shown promising results; however, melanoma cells either possess or acquire resistance to these treatments [1]. Therefore, alternative treatment options are needed to counteract drug resistance. The inhibitor of apoptosis proteins (IAPs), including XIAP (X-linked-IAP), are molecules overexpressed in cancers, where they control apoptosis and inflammation [2]. Melanoma exhibits high levels of XIAP correlating with disease stage and poor treatment outcomes and, besides, modulation of its expression may affect chemotherapy-induced apoptosis in melanoma [3].

However, whether targeting XIAP expression can be exploited therapeutically to synergise with current melanoma-targeted therapies is unknown. To address this, we treated A375 human melanoma cells with combinations of MEK/MAPK inhibitors (Trametinib/Dabrafenib, T + D or Cobimetinib/Vemurafenib, C + V) and an XIAP/IAPs inhibitor (Birinapant; B). The treatments were administered for 7 days. All treatments completely abolished ERK phosphorylation; there were minor differences in overall ERK expression, but XIAP expression remained nearly unchanged (Figure 1A). Compared to naïve cells, double treatment with T + D or C + V alone and combined with B strongly reduced the number of cells in culture, as shown by the phase contrast images (Figure 1B). However, whereas in the treatments T + D and C + V, we detected a small population of surviving cells exhibiting a mesenchymal phenotype previously associated with intrinsic resistance to drugs [4] (white arrows, Figure 1B), additional treatment with B caused these surviving cells to adopt an epithelial-like phenotype, particularly prominent in the C + V treated group (round flattened; Figure 1B). Although we still observed cells, the proliferating EdU-positive cells were visibly reduced in the treatments compared to untreated controls (Figure 1C). The phalloidin-stained cells surviving the triple treatment also displayed an arrangement of actin filaments crossing the cell horizontally and lengthwise. In contrast, these fibres were only lightly visible in the elongated mesenchymal-like cells in the other treatments (Figure 1C). That plasticity of melanoma cells was previously associated with resistance to drugs, and with the expression of markers whose up- or downregulation defined proliferative and invasive cells, including ZEB2, SOX9, SOX10, MITF, AXL, and EGFR [4]. Analysis of these markers in cells undergoing treatments with C + V and C + V + B, which displayed the most remarkable morphological changes, shows similar upregulation of ZEB2 and SOX9, but downregulation of MITF compared to naïve. Further, the expression levels of SOX10 and AXL were enhanced only in the double but not triple-treated cells, and EGFR expression was increased only following the triple treatment with B (Figure 1D). The MITF-low/AXL-high state, detected in the double treatment, is predictive of early resistance to drugs in melanoma [5]. Thus, treatment with B that reduces AXL expression (Figure 1D) may reverse the effect.

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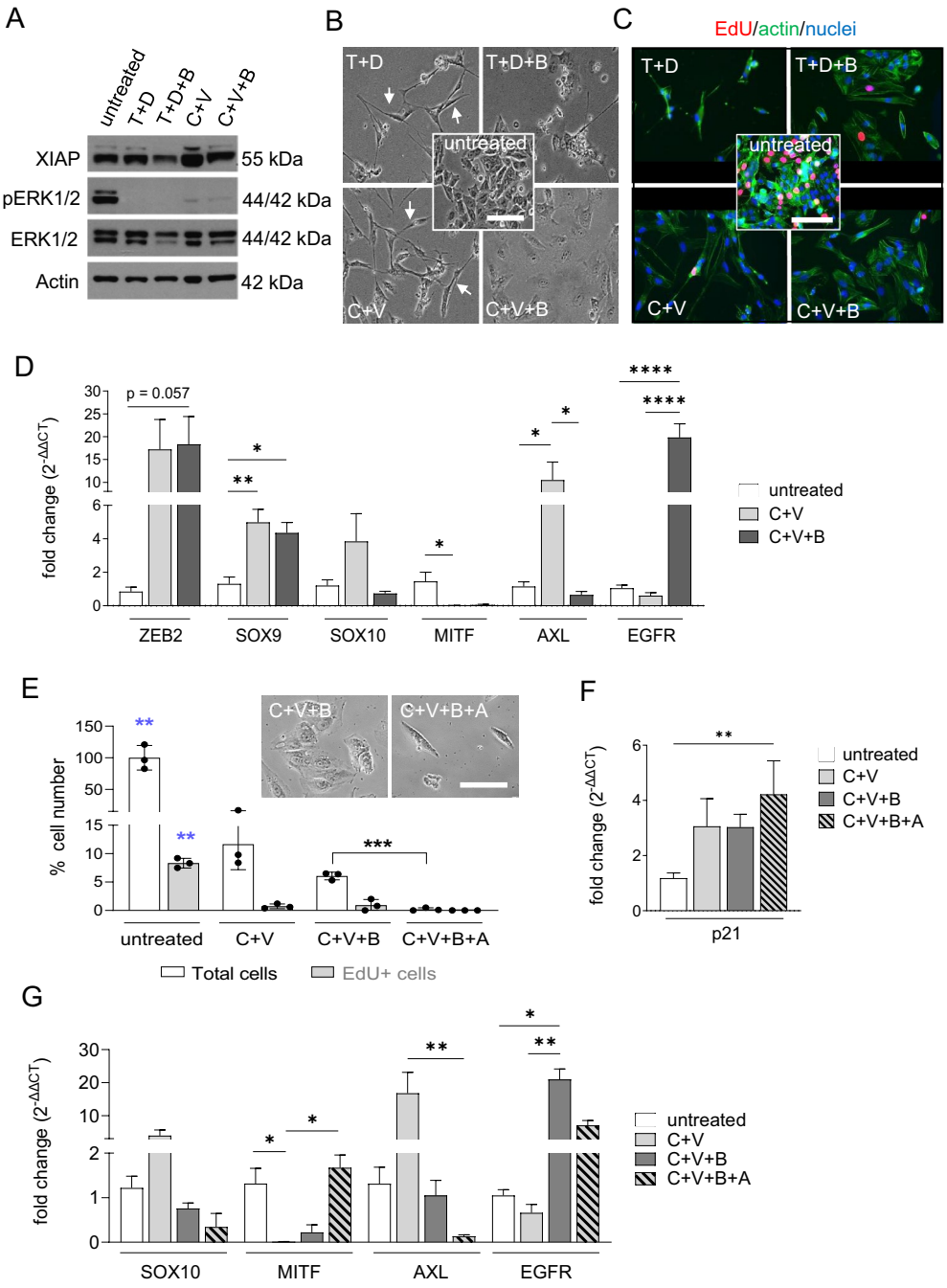

FIGURE 1 | Legend on next page.

Overall, the analysis of the plasticity markers expressed by treated melanoma cells did not highlight a specific change in a more proliferative or invasive population, but underscored the generation of a more heterogeneous population. Interestingly, upon inhibition of BRAF and MEK, the interesting finding is the unexpected regulation of EGFR expression by IAPs in melanoma. EGFR is expressed in less-differentiated melanoma cell lines with epithelioid morphology [6], and its ectopic expression in melanoma cells was shown to cause resistance to BRAFi [7]. In agreement, combined treatment with C+V+B and the EGFRi, Afatinib (A), significantly reduced the total number of viable and proliferating cells surviving the treatments (Figure 1E), and reverted the epithelial-like phenotype visible in the few cells displaying now a more elongated morphology (insert of the graph, Figure 1E). Transcript analysis shows that co-treatment with A leads to significant upregulation of MITF and downregulation of EGFR and AXL, leading to a reversal of the ratio to MITF-high/AXL-low, associated with differentiated and drug-sensitive cells (Figure 1G). Further, the cells surviving all treatments display increased expression of p21, a mediator of cell cycle arrest, compared to untreated cells, suggesting a quiescent state (Figure 1F). The conclusive proof that XIAP may modulate drug resistance in these cells is an exciting possibility requiring further investigation. We could envision a combined treatment approach with Birinapant enhancing drug efficacy by targeting melanoma cells with intrinsic resistance to BRAFi and MEKi. However, this would be difficult to translate into the clinic due to the potential for severe side effects. Besides, further studies are necessary to scrutinise whether melanoma cells pretreated

FIGURE 1 | (A) Protein expression of XIAP, phosphorylated ERK 1/2 (pERK), and ERK 1/2 was analysed in lysates of A375 cells before and after two treatments for 7 days. Drugs used were Trametinib (T) in combination with Dabrafenib (D) or Cobimetinib (C) and Vemurafenib (V), with or without Birinapant (B) (each 1 μM). Actin was detected as a loading control. (B) Phase contrast images of treated A375 cells. Scale bar: 100 μm. (C) Representative images of EdU-positive cells (red) with actin filaments decorated by phalloidin-Alexa 488 of treated A375 cells. Nuclei were stained with Hoechst (Click-iT Plus EdU Imaging Kit C10639, Molecular Probes). Scale bar: 100 μm. (D) mRNA expression of ZEB2, SOX9, SOX10, MITF, AXL and EGFR was analysed in A375 cells treated with C + V or C + V + B, or untreated controls, using quantitative real-time PCR (by the $2^{-\Delta\Delta CT}$ method). RPLP0 (ribosomal protein lateral stalk subunit P0) was used as a reference gene for quantification. Data are presented as mean ± standard error and were analysed by ordinary one-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis test with Dunn's multiple comparison test (*$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$). (E) The cell numbers of A375, treated with the different drug combinations, including Afatinib (A, 1 μM), are displayed as a percentage normalised to the untreated control. The total cell numbers and EdU-positive cells were analysed using Hoechst for nuclei staining and EdU incorporation assays, respectively. Data are presented as mean ± standard deviation and were analysed by unpaired *t*-test (***$p \leq 0.0001$). The significance of the total cells from untreated to all treatments ranged from **$p \leq 0.01$ to ***$p \leq 0.001$, and for the ** EdU-positive cells ***$p \leq 0.001$. In the insert, phase contrast images of A375 cells treated with C + V + B or C + V + B + A. Scale bar: 100 μm. (F) mRNA expression of p21 was analysed in transcripts from A375 cells, untreated and treated, using quantitative real-time PCR (by the $2^{-\Delta\Delta CT}$ method). RPLP0 amplification was used as a reference gene. Data are presented as mean ± standard error and were analysed by ordinary one-way ANOVA with Tukey's multiple comparisons (**$p \leq 0.01$). (G) mRNA expression analysis of SOX10, MITF, AXL and EGFR in un-/treated A375 cells using quantitative real-time PCR (by the $2^{-\Delta\Delta CT}$ method). RPLP0 was used as a reference gene. Data are displayed as mean ± standard error and were analysed by Kruskal-Wallis test with Dunn's multiple comparison test (*$p \leq 0.05$, **$p \leq 0.01$).

with IAP inhibitors and combined with single inhibitors are more tolerable and may provide a better approach.

Author Contributions

P.Z. conceived the study. P.Z. and J.S. designed the experiments. J.S. and N.M. performed the experiments. P.Z., J.S. and H.K. wrote, reviewed, and edited the manuscript. All authors reviewed the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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