



Collagen Prolyl Hydroxylases Are Bifunctional Growth Regulators in Melanoma

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Appropriate post-translational processing of collagen requires prolyl hydroxylation, catalyzed by collagen prolyl 3-hydroxylase and collagen prolyl 4-hydroxylase, and is essential for normal cell function. Here we have investigated the expression, transcriptional regulation, and function of the collagen prolyl 3-hydroxylase and collagen prolyl 4-hydroxylase families in melanoma. We show that the collagen prolyl 3-hydroxylase family exemplified by *Leprel1* and *Leprel2* is subject to methylation-dependent transcriptional silencing in primary and metastatic melanoma consistent with a tumor suppressor function. In contrast, although there is transcriptional silencing of *P4HA3* in a subset of melanomas, the collagen prolyl 4-hydroxylase family members *P4HA1*, *P4HA2*, and *P4HA3* are often overexpressed in melanoma, expression being prognostic of worse clinical outcomes. Consistent with tumor suppressor function, ectopic expression of *Leprel1* and *Leprel2* inhibits melanoma proliferation, whereas *P4HA2* and *P4HA3* increase proliferation, and particularly invasiveness, of melanoma cells. Pharmacological inhibition with multiple selective collagen prolyl 4-hydroxylase inhibitors reduces proliferation and inhibits invasiveness of melanoma cells. Together, our data identify the collagen prolyl 3-hydroxylase and collagen prolyl 4-hydroxylase families as potentially important regulators of melanoma growth and invasiveness and suggest that selective inhibition of collagen prolyl 4-hydroxylase is an attractive strategy to reduce the invasive properties of melanoma cells.

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INTRODUCTION

Melanoma is an aggressive skin cancer with a high metastatic potential. Cytotoxic drugs are rarely effective in melanoma, but targeted agents and immunotherapy have improved the prognosis (Koeblinger et al., 2017; Robert et al., 2015). Notwithstanding these developments, the prognosis for many patients with advanced melanoma remains poor, and novel therapeutic strategies continue to be urgently required.

Alterations in the properties of the basement membrane are a recognized feature of cancer cells. Collagen, the principal structural component (Shoulders and Raines, 2009), must

undergo appropriate post-translational processing for efficient function, and this requires the activities of the prolyl 3-hydroxylases (C-P3Hs) and prolyl 4-hydroxylases (C-P4Hs). The C-P3H Leprecan Like 1 (*Leprel1*, P3H2) has been shown to participate in the processing of basement membrane collagens such as type IV collagen (Fernandes et al., 2011), but the substrate specificity of *Leprel2* is unknown. Like the C-P3H proteins, the C-P4Hs also have important functions in collagen synthesis (Gorres and Raines, 2010; Myllyharju, 2008). In contrast to the C-P3Hs, the C-P4Hs are tetrameric proteins comprising 2 α and 2 β subunits. *P4HA1*, *P4HA2*, and *P4HA3* encode the catalytic α subunits of the three C-P4H

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Abbreviations: C-P3H, collagen prolyl 3-hydroxylase; C-P4H, collagen prolyl 4-hydroxylase; EDHB, ethyl dihydroxybenzoic acid

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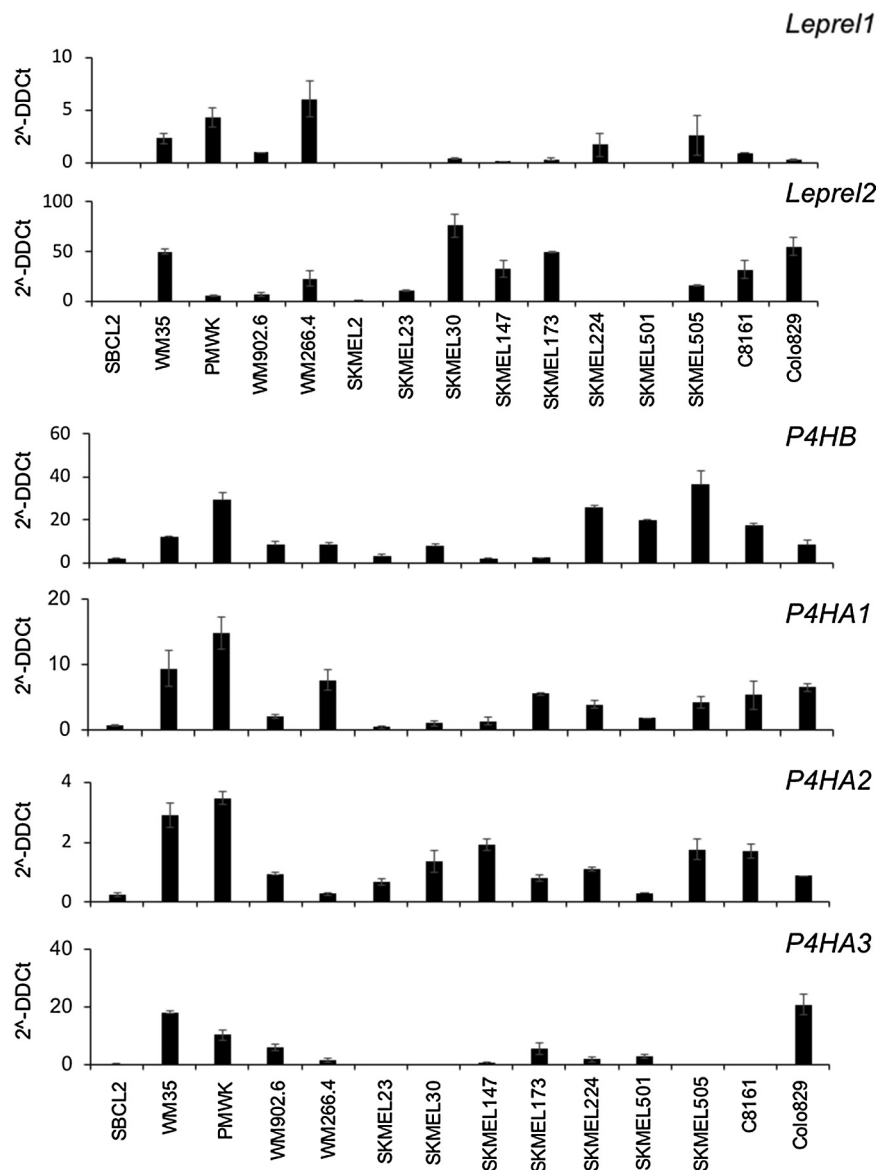


Figure 1. Expression of C-P3H and C-P4H genes in melanoma cell lines. Quantitative PCR analysis of each C-P3H and C-P4H genes in the melanoma cell line panel. cDNA was prepared and quantitative PCR performed as described in Materials and Methods. Expression is shown as $2^{-\Delta\Delta C_t}$ calculated as described in Materials and Methods. Data shown are mean \pm 1 standard deviation. Each experiment was performed in triplicate and repeated at least twice.

proteins, while a common β subunit is encoded by *P4HB*. *P4HA1* and *P4HA2* have been well studied, but little is known of the functions of *P4HA3* since its molecular cloning (Van Den Diepstraten et al., 2003). We have previously shown that C-P3H genes are tumor suppressors, subject to methylation-dependent transcriptional silencing in breast cancer (Shah et al., 2009). In non-Hodgkin's lymphoma, C-P3H and C-P4H genes are silenced by methylation at high frequencies, with distinct patterns of methylation in different lymphoma types (Hatzimichael et al., 2012). *P4HA2* is a direct transcriptional target for p53, suggesting a tumor suppressor function (Teodoro et al., 2006). Conversely, a recent study found *P4HA1* and *P4HA2* to be essential for breast cancer metastasis (Gilkes et al., 2013).

Changes in the genome and epigenome of melanoma cells that drive metastasis remain incompletely described. Evidence from our own and others' studies show that changes in CpG island methylation occur in metastatic melanoma. For example, increased methylation in the CpG island of *TFPI2* is associated with metastatic melanoma (Lo Nigro et al., 2013;

Tanemura et al., 2009), whereas the opposite effect is seen with *NT5E* (Wang et al., 2012).

Here, we have analyzed the expression, regulation, and function of the collagen prolyl hydroxylase genes in melanoma.

RESULTS

C-P3H and C-P4H genes are epigenetically regulated in melanoma cell lines

We examined mRNA expression of the C-P3H (*Leprel1* and *Leprel2*) and C-P4H (*P4HA1*, *P4HA2*, *P4HA3*, and *P4HB*) encoding genes in a panel of melanoma cell lines (Figure 1). *Leprel1* was expressed in the radial growth phase melanoma WM35 and PMWK and in WM266.4 and the metastatic melanoma cell lines SKMEL224, SKMEL505, and C8161, but was undetectable in SBCL2, SKMEL2, SKMEL23, SKMEL147, and SKMEL501 (Figure 1). *Leprel2* was more abundantly expressed across the cell line panel, but not detectable in SBCL2, SKMEL224, and SKMEL501 (Figure 1). *P4HB* (encoding the β subunit common to all C-P4H proteins) was

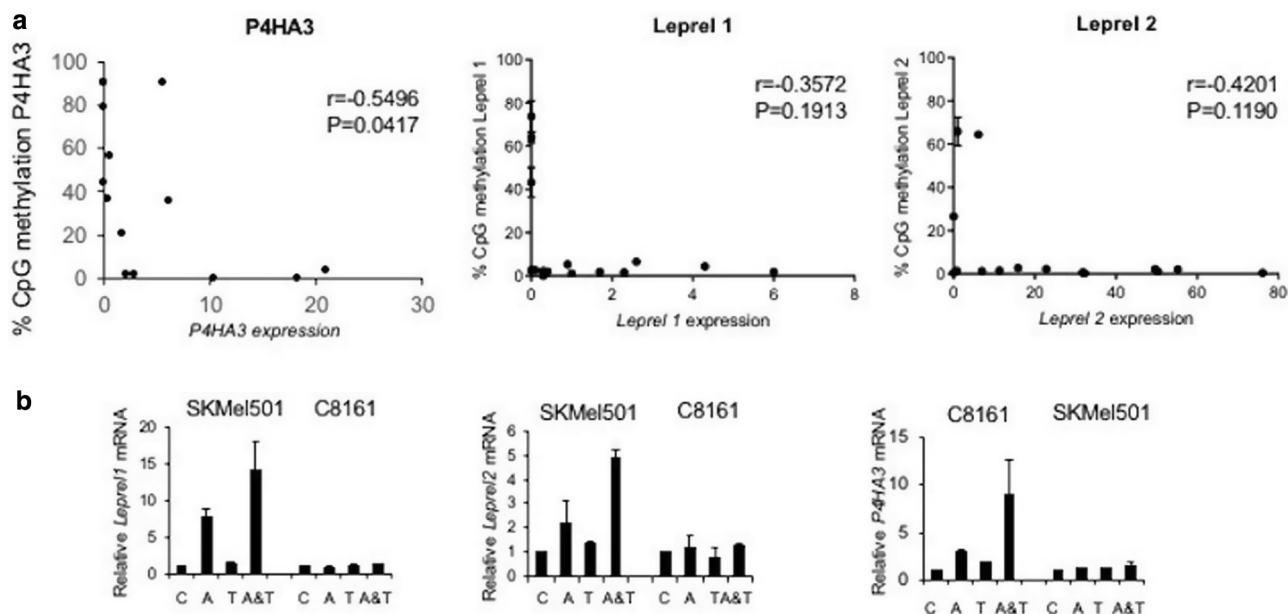


Figure 2. C-P3H and C-P4H are epigenetically regulated in melanoma. (a) Scatter plot showing that methylation in the *P4HA3*, *Leprel1* and *Leprel2* CpG islands is negatively correlated with expression. Data are mean \pm standard error of the mean ($n = 15$). Correlation analysis was done using Pearson's correlation coefficient. (b) Demethylation reactivates expression of *P4HA3* in melanoma cells with CpG island methylation. C8161 and SKMEL501 cells were grown in the presence of azacytidine (A), trichostatin A (T) or both agents (A&T). Expression was determined by quantitative PCR. Data shown are mean \pm 1 standard deviation relative to control cells (C).

detectable in all cell lines (Figure 1). Similarly, *P4HA1* and *P4HA2* mRNA was detectable in all cell lines. In contrast to the other C-P4H encoding genes, *P4HA3* was undetectable in some cell lines (SBCL2 [radial growth phase melanoma], SKMEL23, SKMEL30, SKMEL505, and C8161) and detectable only at very low levels in SKMEL147, SKMEL224, and WM266.4. Of note, in each of these cell lines with undetectable or extremely low expression of *P4HA3*, there was correspondingly moderate to high expression of *P4HA1* and especially *P4HA2* (Figure 1).

A CpG island is located in the upstream regulatory sequences of all examined genes, and we used pyrosequencing to quantitatively test for methylation. Representative pyrograms are shown in Supplementary Figure S1 online and full methylation profiles in Supplementary Figure S2 online. Each gene was unmethylated in normal melanocytes. Dense methylation in *Leprel1* was detected in SKMEL2, SKMEL23, and SKMEL50 and in *Leprel2* in SBCL2, PMWK, and SKMEL50. The *P4HA1* CpG island was uniformly unmethylated and, similarly, *P4HA2* was generally unmethylated, but we did detect methylation in SKMEL23 and SKMEL30. However, this did not correlate with reduced expression (Figure 1). There was, however, a good correlation between methylation in the *P4HA3* CpG island and downregulation of expression with dense methylation in SBCL2, WM902.6, SKMEL505, SKMEL23, SKMEL30, SKMEL147, and C8161. *P4HA3* expression was undetectable in all these lines with the exception of WM902.6, in which expression was reduced relative to unmethylated cell lines, such as Colo829. Correlation analysis demonstrated that expression of *P4HA3* was negatively associated with methylation ($r = -0.5496$; $P = 0.0417$) (Figure 2a). *Leprel1* and *Leprel 2* also demonstrated a negative correlation ($r = -0.3572$ and $r = -0.4201$,

respectively). Although this did not quite reach statistical significance, it is clear that the presence of dense CpG island methylation is associated with low to no expression for both genes. For example, the three cell lines that have dense CpG island methylation in *Leprel1* (SKMEL2, SKMEL23, and SKMEL501), all have undetectable expression (Figure 2a). Similarly, the three cell lines with dense CpG island methylation in *Leprel2* (SBCL2, SKMEL501, and PMWK) have undetectable (SBCL2 and SKMEL501) or low (PMWK) expression (Figure 2a). To further confirm that methylation downregulates expression, we performed demethylation experiments with 5' azacytidine and trichostatin A. C8161 cells (express *Leprel1*, *Leprel2* but do not express *P4HA3* mRNA) and SKMEL501 (do not express *Leprel* or *Leprel2* but do express *P4HA3*) were grown with or without 5' azacytidine and trichostatin A. Quantitative PCR analysis showed that *P4HA3*, but not *Leprel1* and *Leprel2*, was upregulated by 5' azacytidine and 5' azacytidine + trichostatin A in C8161 cells, whereas *P4HA3* was not upregulated in SKMEL501, but both *Leprel1* and *Leprel2* were upregulated (Figure 2b).

C-P3H and C-P4H methylation in primary and metastatic clinical melanoma cases

These results prompted us to analyze methylation in a series of 50 clinical melanoma cases. We used benign pigmented nevi as control tissues. Representative pyrograms are shown in Supplementary Figure S1. Methylation levels for each of the C-P3H and C-P4H genes were uniformly low in benign nevi (Supplementary Figure S3 online). In melanomas, *P4HA1* was unmethylated in all cases, and *P4HA2* mean methylation was $<5\%$. For both *Leprel1* and *P4HA3*, levels of methylation were significantly higher in melanoma than in the benign nevi ($P < 0.001$ for both genes) (Supplementary Figure S3),

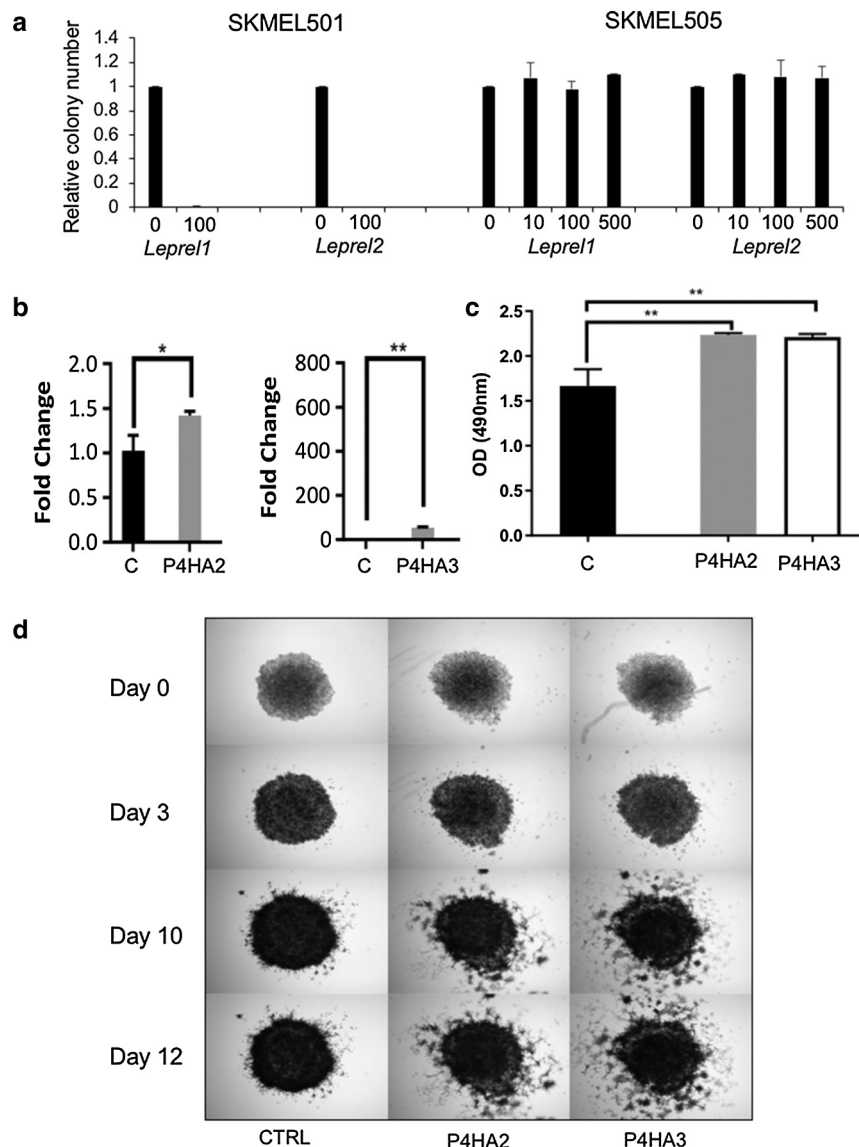


Figure 3. Collagen prolyl 3-hydroxylase and collagen prolyl 4-hydroxylase have opposing effects on melanoma progression. (a) Ectopic expression of *Leprel1* and *Leprel2* in cells lacking endogenous expression blocks proliferation. (b) Quantitative PCR analysis in PMWK engineered to express of P4HA2 and P4HA3. Data are mean \pm standard error of the mean ($n = 3$). Significance was tested using one-way analysis of variance with Dunnett's post-hoc testing. (c) P4HA2 and P4HA3 overexpression significantly increase proliferation in PMWK cells. Graphs shows OD at 490 nm 6 days post treatment. Data are mean \pm standard error of the mean over biological repeats ($n = 2$) performed in triplicate. Significance was tested using two-way analysis of variance with Tukey's post-hoc testing. (d) P4HA2 and P4HA3 overexpression increase the invasiveness relative to control (CTRL) of PMWK early radial growth phase melanoma cells. Images are representative of two independent experiments performed in triplicate. OD, optical density.

showing that CpG island methylation in *Leprel1* and *P4HA3* is specific to malignant melanocytes. We then analyzed *Leprel1* and *P4HA3* methylation in metastatic melanomas. For *Leprel1*, the density of methylation was similar in nodal metastatic lesions and primary lesions, but for *P4HA3* methylation was higher in some lymph node metastases than in primary cases ($P < 0.01$; [Supplementary Figure S3](#)).

C-P3H and C-P4H have opposing effects on melanoma

These results imply that the two major families responsible for post-translational modification of collagen have opposing effects on the properties of melanoma cell lines. In initial studies to validate this hypothesis, we transfected various melanoma cells with expression plasmids for C-P3H and C-P4H and attempted to select clones stably overexpressing the transfected sequences. *Leprel1* and *Leprel2* were overexpressed in SKMEL501 (methylated *Leprel1* and *Leprel2*) and SKMEL505 cells (unmethylated *Leprel1* and *Leprel2*), transfected cells were selected in G418 and colony formation determined after 10–14 days. Whereas colony numbers

(relative to vector only controls) were essentially unaffected by ectopic expression of either gene in SKMEL505, no surviving colonies could be recovered from SKMEL501 cells transfected with either gene ([Figure 3a](#)). The results are consistent with a growth suppressor function for *Leprel1* and *Leprel2*. Next, we tested the effect of overexpressed *P4HA2* and *P4HA3*. We performed these studies in PMWK cells to determine whether changes in C-P4H expression are able to confer a more aggressive phenotype to an early radial growth phase melanoma. Cells were transfected with expression plasmids encoding *P4HA2* and *P4HA3*, colonies selected and overexpression confirmed using quantitative PCR ([Figure 3b](#)). Increased expression of both genes was associated with a significant increase in basal proliferation rate in PMWK cells ([Figure 3c](#)). We then tested whether invasiveness of melanoma cells was affected by ectopic expression of *P4HA2* and *P4HA3* and analysis in a 3-dimensional spheroid invasiveness assay ([Figure 3d](#)). PMWK 3-dimensional tumor spheroids stably expressing ectopic *P4HA2* and *P4HA3* clearly had greater invasive activity than control spheroids

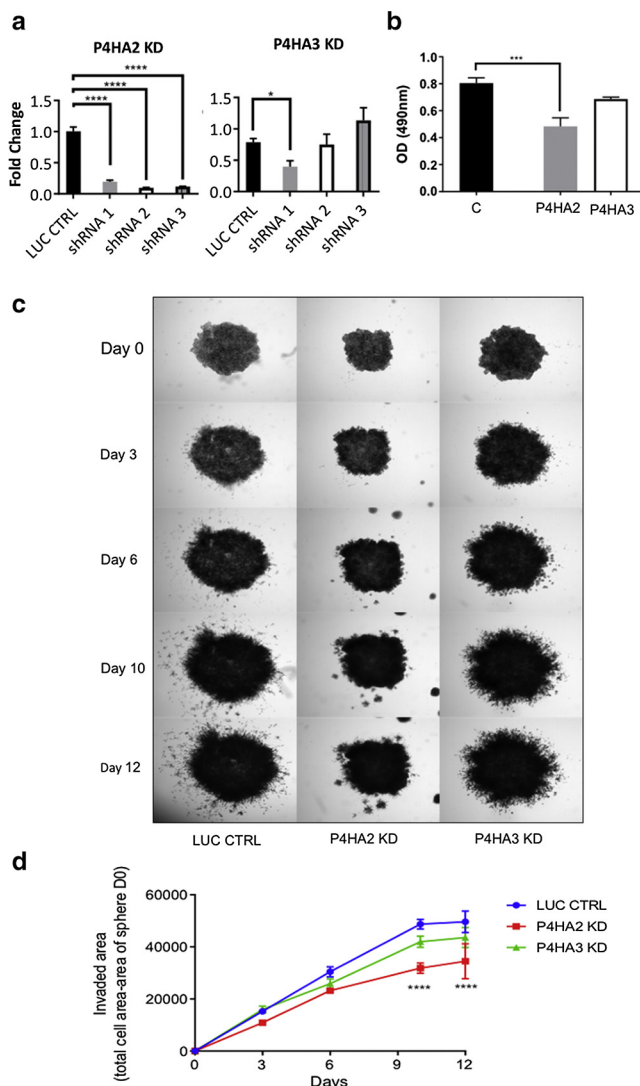


Figure 4. Inhibition of collagen prolyl 4-hydroxylase gene expression reduces melanoma proliferation and invasiveness. (a) Quantitative PCR analysis of *P4HA2* and *P4HA3* in PMWK cells expressing short hairpin RNA. Data are mean $2^{-\Delta\Delta Ct} \pm$ standard error of the mean ($n = 3$). Significance: one-way analysis of variance with Dunnett's post-hoc testing. (b) *P4HA2* and *P4HA3* knockdown reduce proliferation of PMWK cells. Quantification was performed using sulforhodamine b as described in Materials and Methods. Data are mean \pm standard error of the mean over biological repeats ($n = 2$) performed in triplicate. Significance shows the difference to appropriate control, using one-way analysis of variance with Sidak's post-hoc testing. (c) Collagen prolyl 4-hydroxylase knockdown reduces invasiveness of PMWK cells. Representative images are shown. Spheroids were imaged at $\times 2$. (d) Reduced invasion of PMWK 3-dimensional spheroids. Data are mean \pm standard error of the mean ($n = 2$), in triplicate. Significance: two-way analysis of variance with Tukey's post-hoc testing. KD, knockdown; LUC CTRL, luciferase control; shRNA, short hairpin RNA.

(Figure 3d). We next used inhibitory RNA via lentiviral vectors to selectively target gene expression of *P4HA2* and *P4HA3* in PMWK cells and checked knockdown by quantitative PCR (Figure 4a). Knockdown of *P4HA2* caused a significant reduction in proliferation relative to control cells (Figure 4b). In the case of *P4HA3* knockdown clones were obtained with short hairpin RNA1 and proliferation was reproducibly lower than control cells, although this did not reach statistical significance (possibly due to lower levels of

knockdown) (Figure 4a, 4b). Next, we tested the invasive potential of the same PMWK knockdown clones (Figure 4c, 4d). Due to limitations in image thresholding, the inhibitory effect could not be quantified in its entirety. Nonetheless, there was a significant reduction in cancer cell invasion (Figure 4d), and representative images clearly show that *P4HA2* knockdown almost completely abolishes invasive leader cells capable of traveling to sites distant of the sphere (Figure 4c). Similarly, *P4HA3* knockdown was reproducibly associated with reduced invasiveness.

C-P4H inhibition has antiproliferative effects in melanoma via induction of apoptosis

We then test whether pharmacological inhibition of C-P4H affects melanoma cell properties (Vasta and Raines, 2018). We tested two compounds, ethyl dihydroxybenzoic acid (EDHB) and the highly specific and potent C-P4H inhibitor 2-(5-carboxythiazol-2-yl)pyridine-5-carboxylic acid (Vasta et al., 2016). In initial experiments, we titrated the concentration of EDHB testing a range of concentrations from 32 μ M to 512 μ M in PMWK and SKMEL23 cells (Figure 5a). Concentrations of EDHB as low as 128 μ M have a significant inhibitory effect on proliferation of both cell lines (Figure 5b). We then compared the potency of EDHB with 2-(5-carboxythiazol-2-yl)pyridine-5-carboxylic acid in proliferation assays (Figure 5c). 2-(5-Carboxythiazol-2-yl)pyridine-5-carboxylic acid leads to a significantly greater inhibition at various doses in both cell lines, consistent with its greater potency as a pharmacological C-P4H inhibitor (Vasta et al., 2016).

To determine the mechanism of cell death caused by EDHB treatment, PMWK cells were treated with EDHB for 72 hours and analyzed using the Annexin V apoptotic assay (Figure 5d). EDHB caused a significant increase in the proportion of cells in early-stage apoptosis, demonstrating that EDHB functions as an inducer of apoptosis in PMWK cells. We sought to determine whether *P4HA2*, *P4HA3*, or both, are targeted by EDHB to induce apoptosis. We therefore performed the same apoptotic assay on C-P4H knockdown cells (Figure 5d). An increase in apoptosis was seen following EDHB treatment with each sample, regardless of knockdown ($P < 0.0001$). However, *P4HA2* knockdown cells demonstrated significantly fewer cells being induced into early-stage apoptosis compared to the luciferase control EDHB-treated control, and the same was true of *P4HA3* knockdown cells with late-stage apoptotic cells. Taken together, these data suggest that both *P4HA2* and *P4HA3* are targeted by EDHB with resultant induction of apoptosis in PMWK cells.

To confirm the finding that *P4HA2* knockdown resulted in a significant decrease in invasion, we tested the effect of EDHB on invasion in PMWK 3-dimensional spheroids. A clear and significant decrease in invasion was demonstrated, even with low doses of EDHB (Figure 6a, 6b). Again, the limitations in image thresholding render the quantified inhibitory effect as lower than representative images suggest, although notwithstanding this issue, there was a significant inhibition of invasion by EDHB doses as low as 64 μ M (Figure 6a, 6b).

Expression of C-P4H is prognostic in melanoma

Together, these data are consistent with an oncogenic function for the C-P4H family in melanoma and we were

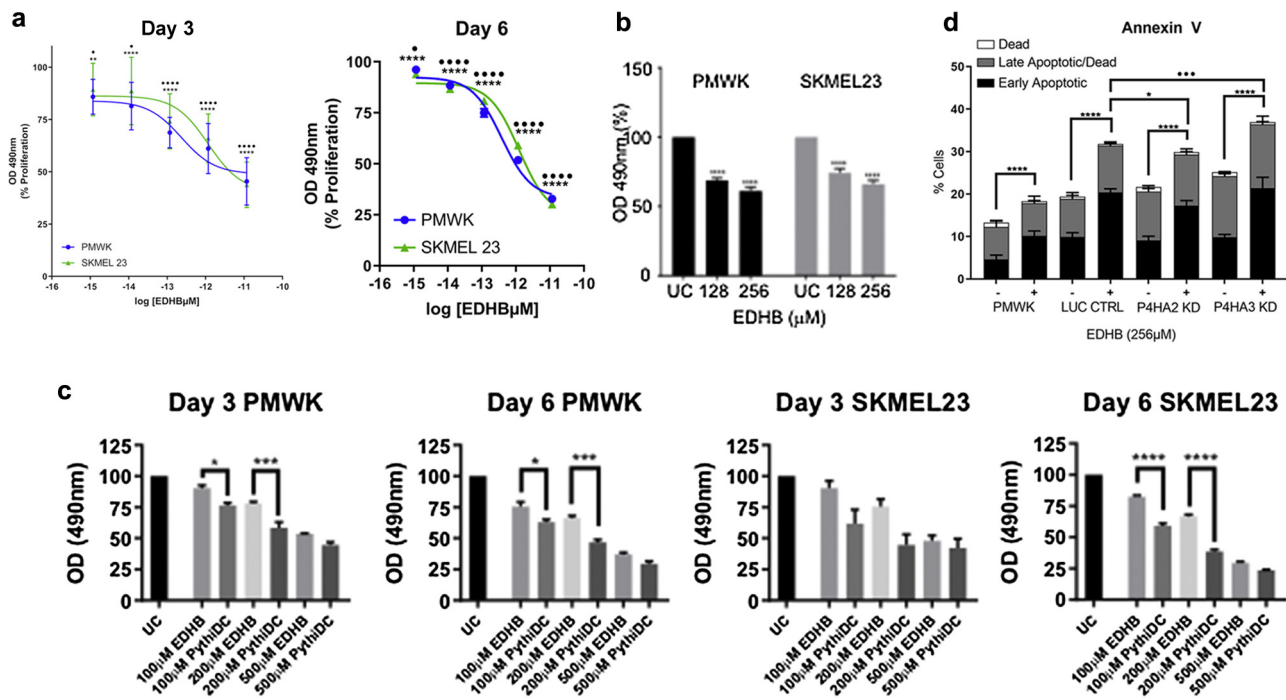


Figure 5. Inhibition of collagen prolyl 4-hydroxylase has antiproliferative effects on melanoma cells via induction of apoptosis. (a, b) Inhibition of proliferation of PMWK (blue) and SKMEL23 (green) by EDHB. Data are mean \pm standard error of the mean over biological repeats ($n = 3$) performed in six replicates. Significance: two-way analysis of variance with Tukey's post-hoc testing. (c) Antiproliferative effect of EDHB and PythiDC on PMWK and SKMEL23. Data are mean \pm standard error of the mean ($n = 2$) in triplicate. Significance: one-way analysis of variance with Sidak's post-hoc testing. (d) EDHB induces apoptosis in PMWK cells. Apoptosis was measured via Annexin V staining on day 3. Data are mean \pm standard error of the mean ($n = 3$). Live cell population not shown. Significance: two-way analysis of variance with Tukey's post-hoc testing. *Differences in early-stage apoptosis. **Differences in late-stage apoptosis. EDHB, ethyl dihydroxybenzoic acid; PythiDC, 2-(5-carboxythiazol-2-yl)pyridine-5-carboxylic acid.

therefore interested to determine whether expression has prognostic utility in melanoma. We interrogated the Jonsson data set via the R2: Genomics Analysis and Visualization Platform (<http://r2.amc.nl>) (Cirenajwis et al., 2015). Disease-free survival was significantly worse in cases overexpressing both *P4HA2* ($P = 0.037$) and *P4HA3* ($P = 0.0064$) (Figure 6c).

DISCUSSION

We present evidence that although the C-P3H encoding genes, *Leprel1* (*P3H2*) and *Leprel2* (*P3H3*), are subject to transcriptional silencing in both melanoma cell lines and clinical cases of melanoma, methylation was observed only in *P4HA3* among the C-P4H genes. To our knowledge, C-P4H (*P4HA3*) gene silencing has not been reported previously in any human non-hematologic cancer, and *Leprel* silencing in solid tumors has only been reported in one previous study (Shah et al., 2009). Of note, the majority of melanomas we analyzed demonstrated CpG island methylation in at least one of *Leprel1* and *Leprel2*, but very rarely were both genes silenced. This suggests that loss of the post-translational collagen modifying functions of the C-P3H family may be important in malignant development in melanoma, but also implies that there may be functional redundancy between P3H family members to preserve one or more critical functions. We also noted that in melanoma cell lines with undetectable or extremely low expression of *P4HA3*, there was usually moderate to high expression of

P4HA1 and especially *P4HA2*, again implying functional redundancy between P4H family members.

There are, however, no mechanistic studies of *P4HA3* in human cancer and no previous reports of the function of either the C-P3H or C-P4H family in melanoma. Although at face value the methylation data imply a tumor suppressor function for *P4HA3*, our detailed mechanistic studies do not support this, as clones stably expressing *P4HA3* are more invasive and proliferative than parental controls and, conversely, knockdown clones are less invasive. Methylation-dependent silencing of genes that have potentially oncogenic functions in melanoma may appear counterintuitive. There are, however, precedents in melanoma and indeed in other cancer types. For example, *NT5E* has definite oncogenic properties and yet is subject to methylation-dependent transcriptional silencing in both breast cancer and metastatic melanoma, with better clinical outcomes in cases with methylation (Lo Nigro et al., 2013; Wang et al., 2012).

To gain mechanistic insight, we modulated expression of *Leprel1*, *Leprel2*, *P4HA2*, and *P4HA3* in melanoma cell lines using ectopic expression and (for *P4HA2* and *P4HA3*) knockdown. Ectopic expression of *Leprel1* and *Leprel2* efficiently abrogated proliferation and colony-forming ability in cell lines in which the endogenous gene is silenced by CpG island methylation. We observed similar effects in breast cancer (Shah et al., 2009). However, clones of the cell line PMWK engineered to stably express *P4HA2* and *P4HA3* (expression confirmed by quantitative PCR) exhibited a

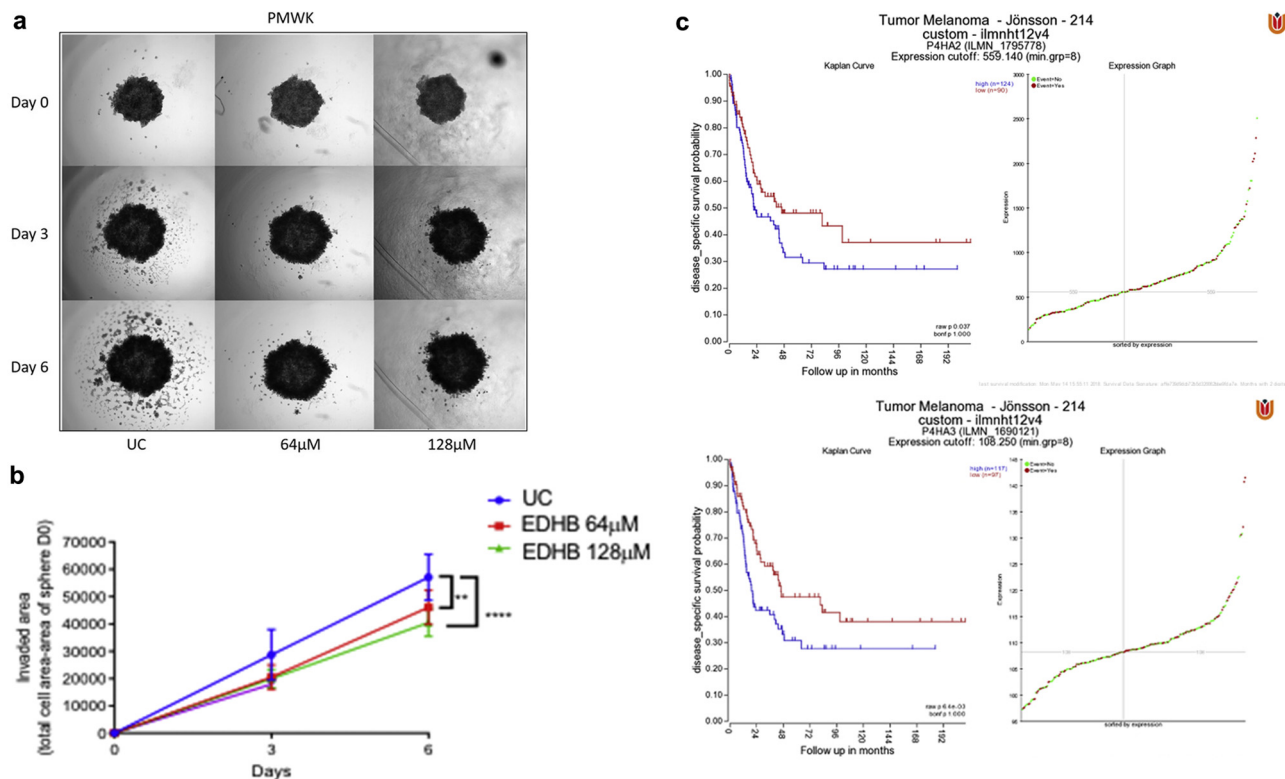


Figure 6. Blocking collagen prolyl 4-hydroxylase inhibits melanoma invasion. (a) EDHB inhibits invasiveness of PMWK 3-dimensional tumor spheroids. Representative images from two independent experiments performed in triplicate. Spheroids were imaged at $\times 2$ on days 0, 3, and 6 post treatment. (b) Quantification of the effect of EDHB on PMWK 3-dimensional spheroid invasion. Spheroids were imaged at $\times 2$ on days 0, 3, and 6 post treatment. Data are mean \pm standard error of the mean ($n = 2$) performed in triplicate. Significance was compared to the appropriate control using two-way analysis of variance with Tukey's post-hoc testing. (c) Overexpression of C-P4H genes is associated with worse disease-free survival in melanoma. Kaplan–Meier plots by gene expression were created using the “Tumor Melanoma-Jönsson-214-custom-ilmnh12v4” data set ($n = 214$) (Cirenajwis et al., 2015). EDHB, ethyl dihydroxybenzoic acid.

higher basal proliferation rate and invasiveness than control and parental PMWK cells, and this finding was further confirmed by knocking down *P4HA2* and *P4HA3* expression and observing the reverse effect. These observations prompted investigation of pharmacologic inhibition of the C-P4H proteins using the C-P4H selective agents EDHB and the highly potent and selective 2-(5-carboxythiazol-2-yl)pyridine-5-carboxylic acid (Vasta et al., 2016). Both agents demonstrated efficient anti-proliferative activity in PMWK and the metastatic melanoma cell line SKMEL23 and we have further shown that EDHB induces apoptosis, which is at least partially dependent on *P4HA2* and *P4HA3* (knockdown of *P4HA2/3* reduced EDHB-induced apoptosis). EDHB also has an anti-invasion action, completely inhibiting migratory leader cells from traveling from the tumor spheroid.

Bioinformatic analysis of a well-validated set of clinical data demonstrated a clear negative correlation between *P4HA2* and *P4HA3* overexpression and time-dependent clinical outcomes, such as disease-free survival.

We have therefore shown, through mechanistic studies and bioinformatics analysis, that overexpression of *P4HA2* and *P4HA3* is capable of promoting malignant progression in melanoma and that this effect is reversible via inhibition of *P4HA2* and/or *P4HA3*. It is likely that this effect is related to the role of *P4HA2* and *P4HA3* in regulating collagen deposition (Xiong et al., 2014). Collagens form the main structural component of the extracellular matrix and are known to

control cellular processes, such as proliferation, invasion, and migration in both cancer progression and in health (Pozzi et al., 1998; Zhang et al., 2013; Provenzano et al., 2008). P4H catalyzes the formation of 4-hydroxyproline, which supports the folding of newly synthesized collagen polypeptides into stable triple helix structures (Nokelainen et al., 2001). Blocking the P4H enzyme prevents maturation of collagens, and their deposition in the extracellular matrix (Sasaki et al., 2012) and it has been shown in breast cancer models that inhibition of P4H leads to a reduction in extracellular collagen deposition along with reduced invasiveness (Xiong et al., 2014). Therefore, the reduction in phenotypic aggression we observed in melanoma cells when P4H is inhibited could be driven by a reduced level of mature collagens with resulting inhibition of invasiveness and potentially angiogenesis. Taken together, our results suggest that P4H is a viable therapeutic target for reducing growth and invasion in melanoma.

MATERIALS AND METHODS

Cell lines, plasmids, and short hairpin RNA

Melanoma cell lines and primary human melanocytes were grown as described previously (Lo Nigro et al., 2013; Wang et al., 2012). Plasmids encoding *Leprel1* and *Leprel2* were described previously (Shah et al., 2009). To generate a *P4HA2* expression vector, the ORF was purchased from Origene and subcloned into pcDNA3-DEST40 using Invitrogen Gateway Technology to generate pcDNA3.2/V5/

GW/P4HA2. pcDNA3.2/V5/GW/CAT served as the control plasmid. The sequence of both plasmids was verified by sequencing. pcDNA3P4HA3 was described previously (Van Den Diepstraten et al., 2003). Short hairpin RNA sequences were obtained from the RNAi Consortium (Moffat et al., 2006). These were inserted into EcoRI and AgeI cut pLKO.1–TRC cloning vector (Addgene #10878) and subsequently these plasmids were used for lentiviral particle production and target cell transduction following the Addgene protocol for lentiviral transduction of mammalian cells (<https://www.addgene.org/tools/protocols/plko/#A>). Cell proliferation was measured using the sulforhodamine b colorimetric assay.

Gene expression analysis

The expression of C-P4H genes was determined by quantitative PCR. cDNA was generated from 1 µg RNA using Moloney Murine Leukemia Virus reverse transcriptase (Sigma, St Louis, MO), and 0.5 µg random primer. PCR was performed in 96-well plates using SYBER Select Master Mix, and 0.5 µM primer pairs targeting each mRNA transcript of interest (ThermoFisher Scientific, Waltham, MA). cDNA (50 ng) was added to each well, containing 10 µl SYBER Select, 3 µl nuclease-free water, and the primer pairs. mRNA levels were determined by normalizing to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase and using the relative quantification method (2- $\Delta\Delta C_t$). Data are presented as fold-change compared to housekeeping gene.

Annexin V assay

Two hundred thousand cells were plated in six-well plates and allowed to attach overnight. The following day, cells were treated with appropriate drug or vehicle control. On day 3, wells were harvested via trypsinization and stained using the Annexin V Apoptosis Detection kit, according to manufactures direction (ThermoFisher). Cells were analyzed on a Muse Cell Analyzer (Millipore Sigma, Burlington, MA).

Spheroid 3-dimensional invasion assay

Invasion was measured by generating 3-dimensional tumor spheroids and measuring their invasion into surrounding growth factor reduced Matrigel (Corning, Tewksbury, MA). Five thousand cells per well were plated in a low attachment 96-well plate with 200 µl media. Following 4 days growth, cells were visually analyzed to confirm spheroid formation.

Colony-forming assays

The 2×10^5 cells/6-cm dish were transfected after 24 hours with varying amounts of expression plasmids for *Leprel1*, *Leprel2*, *P4HA2*, and *P4HA3* or control vector using Metafectene (Biontex, München, Germany) according to the manufacturer's instructions. Forty-eight hours after transfection, G418 (400 ng/ml) was added and cells were monitored daily by light microscopy for the appearance of colonies, which were counted by staining with Coomassie Brilliant Blue. Cell clusters of 50 cells were defined as colonies.

Clinical material

The study was approved by The Tayside Tissue Bank, under delegated authority from the Tayside Local Research Ethics Committee, with written informed consent from patients. We analyzed 50 clinical cases of melanoma, including primary and metastatic lesions. In all cases, microdissection of tissue sections was performed to enrich for melanoma cells prior to isolation of nucleic acids. Benign pigmented nevi from sun-exposed skin were control tissues. We used proteinase K digestion to isolate genomic DNA from tissue sections.

Methylation analysis

Methylation in the CpG islands of the *C-P3H* and *C-P4H* gene families was determined using pyrosequencing with the primers and reaction conditions described previously (Hatzimichael et al., 2012).

Statistics

Statistical analyses were performed using Prism 7 software (Graph-Pad, La Jolla, CA). Significance was as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Kaplan–Meier plots by gene expression were created on the R2: Genomics Analysis and Visualization Platform (<http://r2.amc.nl>) using the “Tumor Melanoma-Jönsson-214-custom-ilmnht12v4” data set (n = 214) (Cirenajwis et al., 2015). The expression cutoff was determined using the Kaplan scan modus, which generates a Kaplan–Meier Plot based on the most optimal mRNA cut-off expression level to discriminate between a good and bad prognosis cohort.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2018.10.038>.

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