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The collagen prolyl hydroxylases are bifunctional growth regulators in melanoma

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Running title: C-P4H in melanoma.

Abbreviations: AZA: Azacytidine; C-P3H: collagen prolyl 3-hydroxylase; C-P4H: collagen prolyl 4-hydroxylase; DHB: Dihydroxybenzoic acid; EDHB: diethyl pythiDC: 2-(5-carboxythiazol-2-yl)pyridine-5-carboxylic acid; Ethyl dihydroxybennzoic acid; RGP: radial growth phase melanoma; SSM: Superficial Spreading melanoma; TSA: Trichostatin A; VGP: Vertical Growth Phase melanoma.

Abstract

Appropriate post-translational processing of collagen requires prolyl hydroxylation, catalyzed by the prolyl 3- (C-P3H) and prolyl 4- (C-P4H) hydroxylases is essential for normal cell function. Here we have investigated the expression, transcriptional regulation and function of the *C-P3H* and *C-P4H* families in melanoma. We show that the CP3H family exemplified by *Leprel1* and *Leprel2* are subject to methylation-dependent transcriptional silencing in primary and metastatic melanoma consistent with a tumour suppressor function. In contrast, although there is transcriptional silencing of *P4HA3* in a sub-set of melanomas, the CP4H family members *P4HA1*, *P4HA2* and *P4HA3* are often over-expressed in melanoma, expression being prognostic of worse clinical outcomes. Consistent with tumour 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 C-P4H inhibitors reduces proliferation and inhibits invasiveness of melanoma cells. Together, our data identify the C-P3H and C-P4H families as potentially important regulators of melanoma growth and invasiveness and suggest that selective inhibition of C-P4H is an attractive strategy to reduce the invasive properties of melanoma cells.

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 prognosis (Robert et al., 2015; Koeblinger et al., 2017). Notwithstanding these developments, 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 prolyl 4-hydroxylases also have important functions in collagen synthesis (Myllyharju, 2008; Gorres and Raines, 2010). 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 proteins, whilst 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 tumour suppressors, subject to methylation-dependent transcriptional silencing in breast cancer (Shah et al., 2009). In non-Hodgkin's lymphoma (NHL) *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 tumour 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 (Tanemura et al., 2009; Lo Nigro et al., 2013) whereas the opposite effect is seen with *NT5E* (Wang et al., 2012).

Here, we have analysed 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 RGP 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 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 (RGP 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 *P4HA*1 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 1 and full methylation profiles in Supplementary Figure 2. 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 down-regulation 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

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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 3 cell lines which have dense CpG island methylation in *Leprel1* (SKMEL2, SKMEL23 and SKMEL501) all have undetectable expression (Figure 2a). Similarly, the 3 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 (AZA) and trichostatin A (T). 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 (AZA) and T. qPCR analysis showed that *P4HA3* but not *Leprel1* and *Leprel2* was up-regulated by AZA and AZA+T in C8161 cells, whereas *P4HA3* was not upregulated in SKMEL501 but both *Leprel1* and *Leprel2* were up-regulated ((Figure 2b).

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

These results prompted us to analyse methylation in a series of 50 clinical melanoma cases. As control tissues we used benign pigmented nevi. Representative pyrograms are shown in Supplementary Figure 1. Methylation levels for each of the *C-P3H* and *C-P4H* genes were uniformly low in benign nevi (Supplementary Figure 3). In melanomas, *P4HA1* was unmethylated in all cases and *P4HA2* mean methylation was less than 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 3) showing that CpG island methylation in *Leprel1* and *P4HA3* is specific to malignant melanocytes. We then analysed *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 3).

CP3H and CP4H 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 CP3H and CP4H and attempted to select clones stably over-expressing the transfected sequences. *Leprel1* and *Leprel2*

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were over-expressed 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 over-expressed *P4HA2* and *P4HA3*. We performed these studies in PMWK cells to determine whether changes in C-P4H expression is 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 over-expression confirmed using qPCR (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 3D spheroid invasiveness assay (Figure 3d). PMWK 3D tumour spheroids stably expressing ectopic *P4HA2* and *P4HA3* clearly had greater invasive activity than control spheroids (Figure 3d). We next used inhibitory RNA (RNAi) via lentiviral vectors to selectively target gene expression of *P4HA2* and *P4HA3* in PMWK cells and checked knock-down by qPCR (Figure 4a). Knock down of *P4HA2* caused a significant reduction in proliferation relative to control cells (Figure 4b). In the case of *P4HA3* knock-down clones were obtained with shRNA1 and proliferation was reproducibly lower than control cells, although this did not reach statistical significance (possibly due to lower levels of knock down, Figure 4a and Figure 4b). Next, we tested the invasive potential of the same PMWK knock down clones (Figure 4c and Figure 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 travelling to sites distant of the sphere (Figure 4c). Similarly, *P4HA3* knockdown was reproducibly associated with reduced invasiveness.

C-P4H inhibition has anti-proliferative 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 (diethyl pythiDC;

Vasta et al., 2016). In initial experiments, we titrated the concentration of EDHB testing a range of concentrations from 32μM to 512μM in PMWK and SKMEL23 cells (Figure 5a). Concentrations of EDHB as low as 128μM have a significant inhibitory effect on proliferation of both cell lines (Figure 5b). We then compared the potency of EDHB with diethyl pythiDC in proliferation assays (Figure 5c). Diethyl pythiDC 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 analysed 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 LUC EDHB treated control, and the same was true of *P4HA3* knockdown cells with late stage apoptotic cells. Taken together, this data suggests 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 3D spheroids. A clear and significant decrease in invasion was demonstrated even with low doses of EDHB (Figure 6a and Figure 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 64μM (Figure 6a and Figure 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 therefore interested to determine whether expression has prognostic utility in melanoma. We interrogated the Jonsson dataset via the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) (Cirenajwis et al., 2015). Disease-free survival was significantly worse in cases over-expressing both *P4HA2* (p=0.037) and *P4HA3* (p=0.0064) (Figure 6c).

Discussion

We present evidence that whereas 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 previously been reported in any human non-haematological cancer, and *Leprel* silencing in solid tumours has only been reported in one previous study (Shah et al., 2009). Of note, the majority of melanomas we analysed 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 *P4HA*1 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 tumour 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, knock-down clones are less invasive. Methylation-dependent silencing of genes which have potentially oncogenic functions in melanoma may appear counter-intuitive. 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., 2012; 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*) knock-down. 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 qPCR) exhibited a 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 pharmacological inhibition of the C-P4H proteins using the C-P4H selective agents EDHB and the highly potent and selective diethyl pythiDC (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 tumour spheroid.

Bioinformatic analysis of a well validated set of clinical data demonstrated a clear negative correlation between P4HA2 and P4HA3 over-expression 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 catalyses the formation of 4-hydroxyproline, which supports the folding of newly synthesised collagen polypeptides into stable triple helix structures (Nokelainen et al., 2001). Blocking the P4H enzyme prevents the 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 shRNA

Melanoma cell lines and primary human melanocytes were grown as described previously (Wang et al., 2012; Lo Nigro et al., 2013). 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 sub-cloned 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). shRNA sequences were obtained from the RNAi Consortium (The RNAi Consortium and 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 (SRB) colorimetric assay.

Gene expression analysis

The expression of CP4H genes was determined by qPCR. cDNA was generated from 1µg RNA using Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT, Sigma), and 0.5µg random primer. PCR was performed in 96-well plates using SYBER Select Master Mix, and 0.5µM primer pairs targeting each mRNA transcript of interest (Thermofisher). 50ng cDNA was added to each well, containing 10µl SYBER Select, 3µl nuclease free water and the primer pairs. mRNA levels were determined by normalising to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) and using the relative quantification method (2-ΔΔCt). Data are presented as fold-change compared to housekeeping gene.

Annexin V Assay

200,000 cells were plated in 6-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 analysed on a Muse Cell Analyser.

Spheroid 3D Invasion Assay

Invasion was measured by generating 3D tumour spheroids and measuring their invasion into surrounding growth factor reduced matrigel (Corning). 5000 cells per well were plated in a low attachment 96-well plate with 200µl media. Following 4 days growth, cells were visually analysed to confirm spheroid formation.

Colony forming assays

$2x10^5$ cells / 6cm dish and transfected after 24h with varying amounts of expression plasmids for *Leprel1*, *Leprel2*, *P4HA2* and *P4HA3* or control vector using Metafectine (Biontex) according to the manufacturer's instructions. 48 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 analysed 50 clinical cases of melanoma including primary and metastatic lesions. In all cases, micro-dissection 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 (GraphPad software, Inc., La Jolla, CA, USA). Significance was taken 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" dataset (n=214) (Cirenajwis et al., 2015). The expression cut-off 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 report no conflicts of interest.

Acknowledgements

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Figure Legends

Figure 1

Expression of *C-P3H* and *C-P4H* genes in melanoma cell lines. qPCR analysis of each *C-P3H* and *C-P4H* genes in the melanoma cell line panel. cDNA was prepared and qPCR performed as described in Methods. Expression is shown as 2^-DDCt calculated as described in Methods. Data shown are means +/- 1SD. Each experiment was performed in triplicate and repeated at least twice.

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 show mean ± SEM (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 qPCR. Data shown are means (+/- 1 SD) relative to control cells (C).

Figure 3

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CP3H and CP4H have opposing effects on melanoma progression. A: Ectopic expression of *Leprel1* and *Leprel2* in cells lacking endogenous expression blocks proliferation. B: qPCR analysis in PMWK engineered to express of P4HA2 and P4HA3. Data shows mean ± SEM (n=3). Significance was tested using one-way ANOVA with Dunnett's post hoc testing. C: *P4HA2* and *P4HA3* overexpression significantly increase proliferation in PMWK cells. Graphs shows OD_{490} 6 days post treatment. Data shows mean ± SEM over biological repeats (n=2) performed in triplicate. Significance was tested using two-way ANOVA with Tukey's post hoc testing. D: *P4HA2* and *P4HA3* overexpression increase the invasiveness relative to control (CTRL) of PMWK early RGP melanoma cells. Images are representative of two independent experiments performed in triplicate.

Figure 4

Inhibition of CP4H gene expression reduces melanoma proliferation and invasiveness. A: qPCR analysis of *P4HA2* and *P4HA3* in PMWK cells expressing shRNA. Data are mean $2^{\wedge}$-DDCt ± SEM (n=3). Significance: one-way ANOVA with Dunnett's post hoc testing. B: *P4HA2* and *P4HA3* knockdown reduce proliferation of PMWK cells. Quantification was performed using SRB as described in Methods. Data shows mean ± SEM over biological repeats (n=2) performed in triplicate. Significance shows the difference to appropriate control, using one-way ANOVA with Sidak's post hoc testing. C: C-P4H knockdown reduces invasiveness of PMWK cells. Representative images are shown. Spheroids were imaged at 2X. D: Reduced invasion of PMWK 3D spheroids. Data shows mean ± SEM (n=2), in triplicate. Significance: two-way ANOVA with Tukeys post hoc testing.

Figure 5

Inhibition of CP4H has anti-proliferative effects on melanoma cells via induction of apoptosis. A and B: Inhibition of proliferation of PMWK (blue) and SKMEL23 (green) by EDHB. Data shown are mean ± SEM over biological repeats (n=3) performed in 6 replicates. Significance: two-way ANOVA with Tukeys post hoc testing. C: Anti-proliferative effect of EDBH and diethyl pythiDC on PMWK and SKMEL23. Data are mean ± SEM (n=2) in triplicate. Significance: one-way ANOVA with Sidak's post hoc testing. D: EDHB induces apoptosis in PMWK cells. Apoptosis was measured via Annexin V staining on day 3. Data shows mean ± SEM (n=3). Live cell population not shown. Significance: two-

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way ANOVA with Tukeys post hoc testing. * differences in early stage apoptosis. • differences in late stage apoptosis.

Figure 6

Blocking CP4H inhibits melanoma invasion. A: EDHB inhibits invasiveness of PMWK 3D tumour spheroids. Representative images from two independent experiments performed in triplicate. Spheroids were imaged at 2X on days 0, 3 and 6 post treatment. B: Quantification of the effect of EDHB on PMWK 3D spheroid invasion. Spheroids were imaged at 2X on days 0, 3 and 6 post treatment. Data shows mean ± SEM (n=2) performed in triplicate. Significance was compared to the appropriate control using two-way ANOVA with Tukeys post hoc testing. C: Over-expression 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 - ilmnht12v4" dataset (n=214) (Cirenajwis et al., 2015).
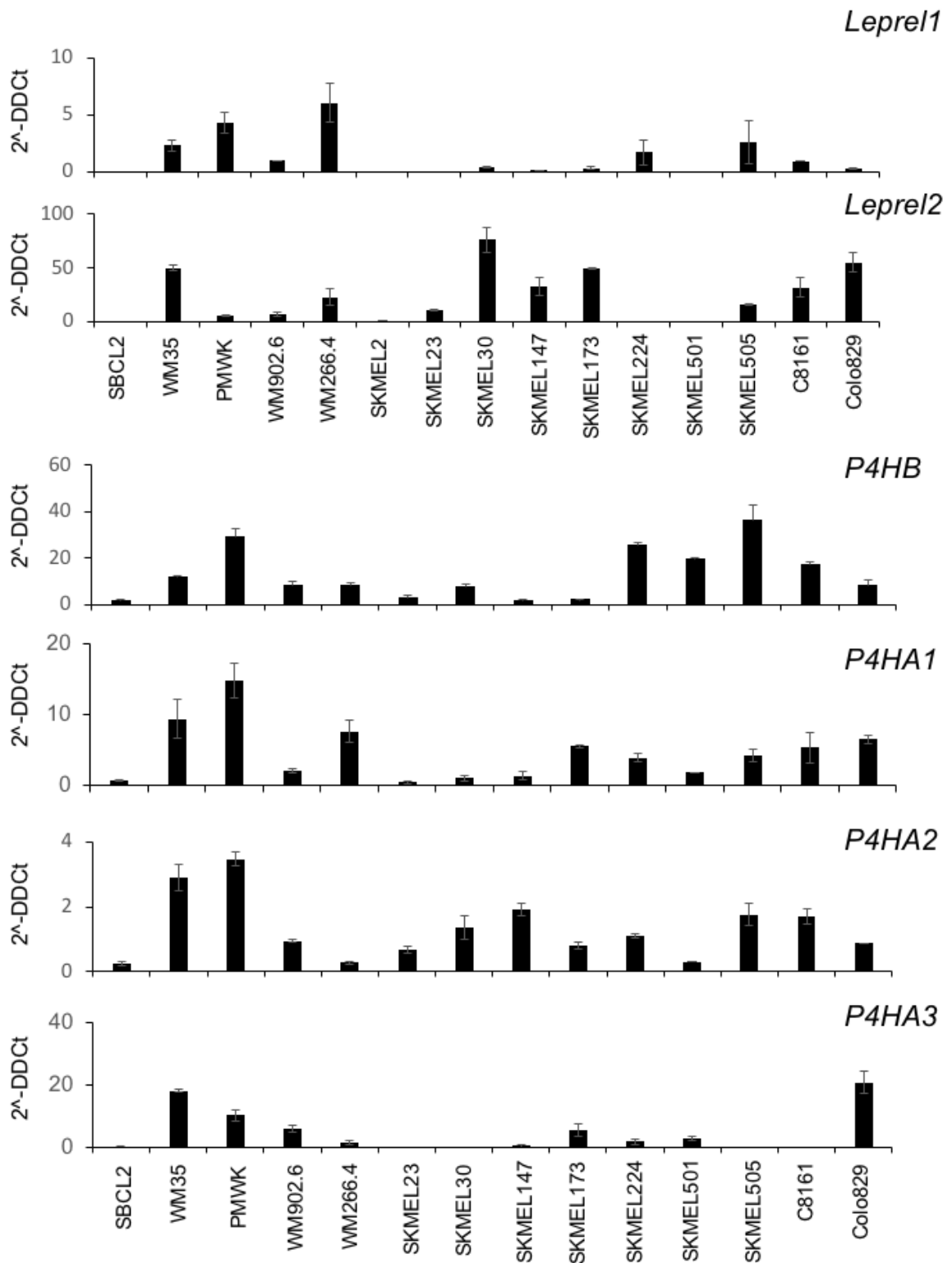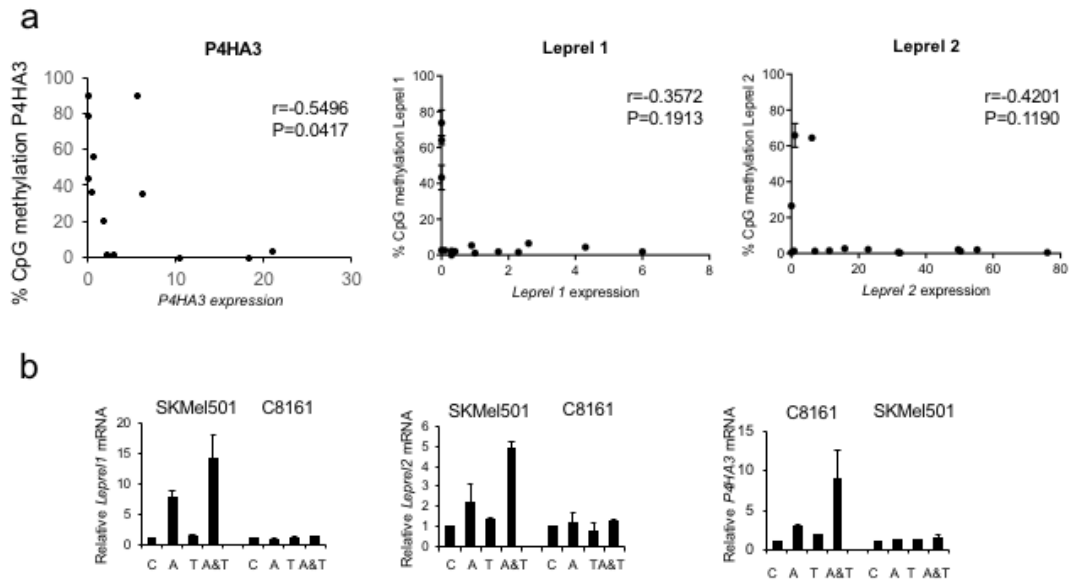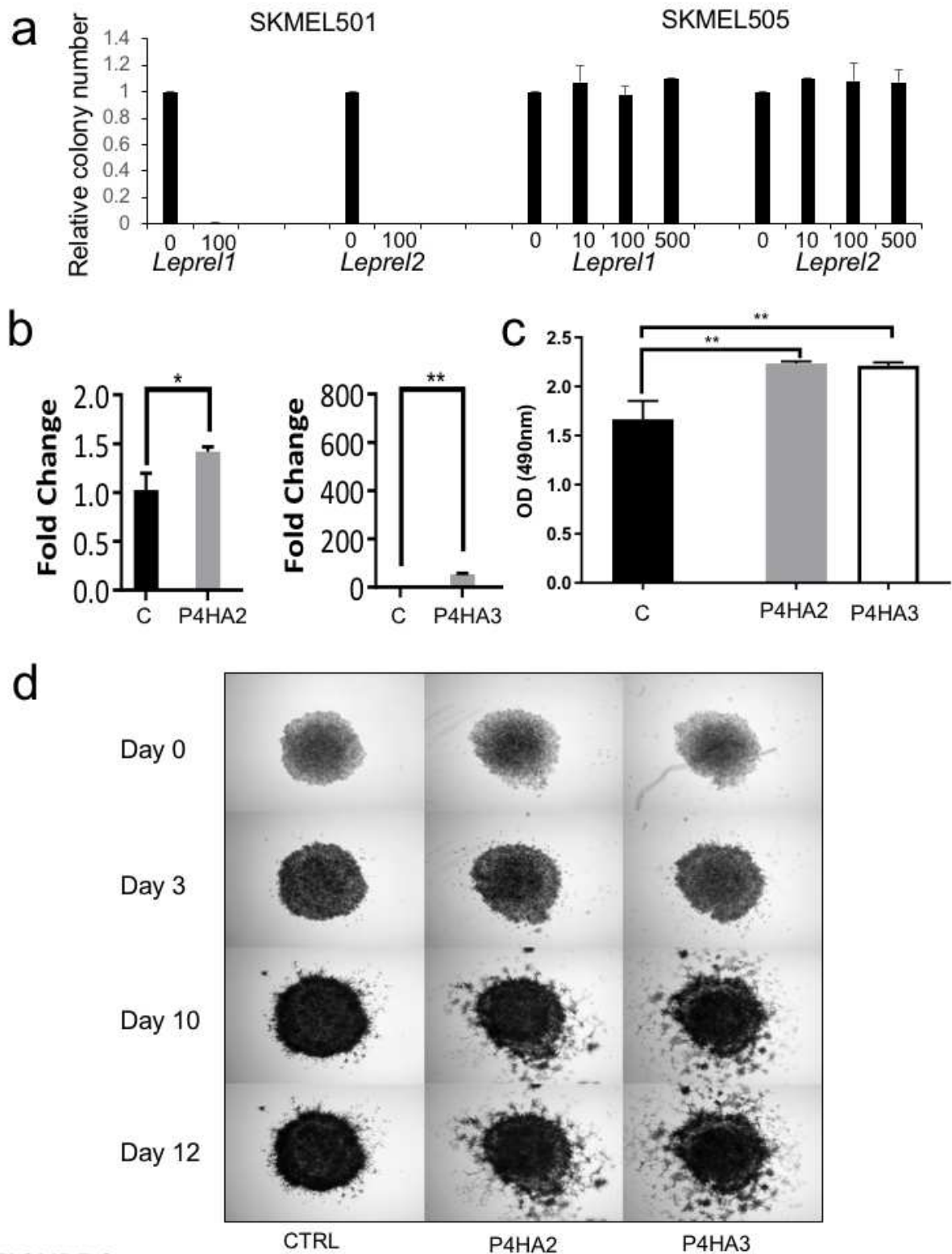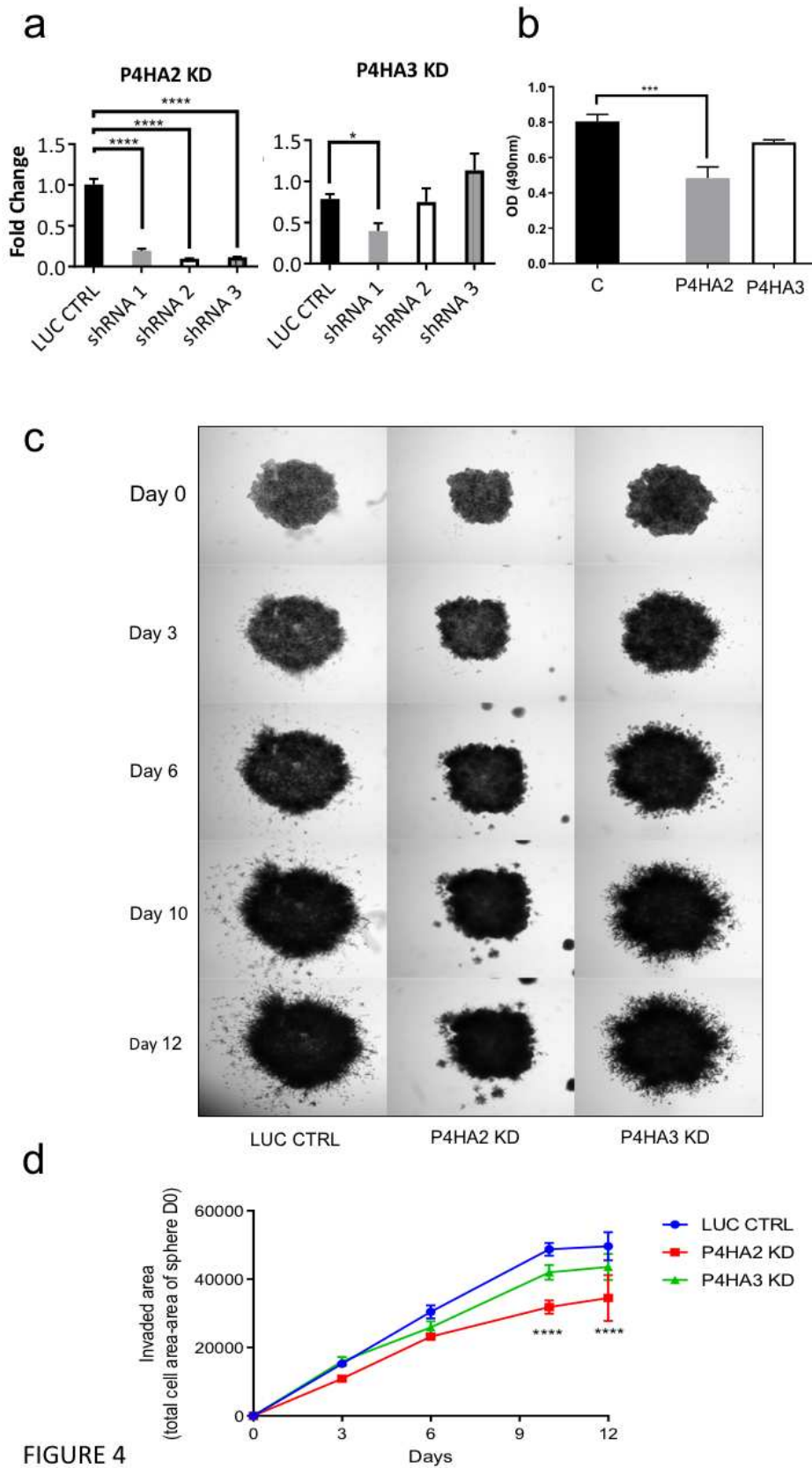
FIGURE 1

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