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UV radiation promotes melanoma dissemination mediated by the sequential reaction axis of cathepsins - TGF-β1 - FAP-α

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Running title: UV induces fibroblast activation protein-α

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Abbreviations: ECM, extracellular matrix; FAP-α, fibroblast activation protein-α; TGF-β1, transforming growth factor-β1; UVR, ultraviolet radiation.

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Abstract

**Background:** Ultraviolet radiation (UVR) is the major risk factor for development of malignant melanoma. Fibroblast activation protein (FAP)-α is a serine protease expressed on the surface of activated fibroblasts promoting tumour invasion through extracellular matrix degradation (ECM). The signaling mechanism behind the upregulation of FAP-α is not yet completely revealed.

**Methods:** Expression of FAP-α was analysed after UVR exposure in *in vitro* co-culture systems, gene expression arrays and artificial skin constructs. Cell migration and invasion was studied in relation to cathepsin activity and secretion of transforming growth factor (TGF)-β1.

**Results:** FAP-α expression was induced by UVR in melanocytes of human skin. The FAP-α expression was regulated by UVR-induced release of TGF-β1 and cathepsin inhibitors prevented such secretion. In melanoma cell culture models and in a xenograft tumour model of zebrafish embryos, FAP-α mediated ECM degradation and facilitated tumour cell dissemination.

**Conclusion:** Our results provide evidence for a sequential reaction axis from UVR via cathepsins, TGF-β1 and FAP-α expression promoting cancer cell dissemination and melanoma metastatic spread.

**Keywords:** FAP-α, UV radiation, melanoma, fibroblast, senescence, invasion, cathepsins, TGF-β1.
Introduction

Ultraviolet radiation (UVR) is the major risk factor for development of cutaneous malignant melanoma, but the role of UVR in melanoma progression and its tendency to metastasize is not studied in detail (Venza et al., 2015). Nevi consist of growth arrested melanocytes that share similarities with senescent cells, and senescence could be referred to as a tumour suppressor mechanism. Histologically, melanocytic nevi precursors can be identified in at least half of all cutaneous melanomas (Tsao et al., 2003). Importantly, several of the features that distinguish benign nevi from melanoma lies in the senescence pathways (Ross et al., 2011). Thus, comparing the gene- and protein expression between nevi and melanoma might provide important information on which signaling pathways and proteins that need to be activated to accomplish the transition of growth arrested cells into a (pre)malignant state (Orfanidis et al., 2016). The determinants of tumour invasion and metastasis include changes in the attachment of cells to their microenvironment, activation of extracellular proteases and modification of the surrounding stromal tissue through direct contact or cell-to-cell crosstalk by soluble mediators. FAP-α (seprase/antiplasmin-cleaving enzyme/dipeptidyl prolyl peptidase 5) is a plasma membrane serine protease and has been detected in the reactive stroma of melanocytic nevi and melanoma (Huber et al., 2003; Scanlan et al., 1994). FAP-α exhibits both protease and collagenase activity and is important for extracellular matrix (ECM) degradation and modification (O'Brien & O'Connor, 2008; Wäster et al., 2011).

Previously, we presented the novel finding that UVR upregulates the expression of FAP-α in melanocytes and melanoma cells in vitro, which facilitates ECM degradation (Wäster et al., 2011). Moreover, we have established that growth and invasion of primary melanoma cells is dependent on secretion of the lysosomal cathepsins B and D (Eding et al., 2015). Overexpression of cathepsins is associated with increased metastatic potential as well as poor prognosis (Palermo & Joyce, 2008; Vasiljeva et al., 2007). Yin et al., revealed that melanoma invasion was enhanced by cathepsin B induced TGF-β1 expression resulting in activated fibroblasts with increased ability to promote
invasion of melanoma (Yin et al., 2012). TGF-β subtypes are constitutively expressed by primary melanoma and metastatic melanoma cells (Lazar-Molnar et al., 2000). Furthermore, TGF-β1 released from melanoma cells stimulates FAP-α expression in surrounding fibroblasts (Wäster et al., 2011).

Given that UVR is important for the initiation and progression of melanoma, the aim of the current study is to identify signaling pathways that contribute to these processes. Specifically, we investigate the relationship between cathepsins, TGF-β1 and FAP-α during UVR exposure using in vitro co-culture systems, ex vivo skin, artificial skin constructs and a xenograft tumour model of zebrafish embryos.
Materials and Methods

Cell cultures and additions

All experiments were performed according to the ethical principles of the Helsinki declaration and were approved by the Ethical Review Board at Linköping University, Sweden. Primary melanocytes, keratinocytes and fibroblasts were obtained from Caucasian donors by means of foreskin circumcisions (0-3 years of age; parental written informed consent) as described previously (Larsson et al., 2005). Four primary melanoma cell lines WM115, WM793, WM278, and FM55P (Wistar Institute, Philadelphia, USA) were used. The fibroblasts and melanoma cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Prior to experiments, cells were trypsinated and seeded at 2.5 x 10⁴ cells/cm².

The following antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were used for inhibition when indicated; anti-TGF-β1 (200 ng/ml), anti-FAP-α (20.6 µg/ml), or anti-cathepsin D (42 µg/ml; Athens Research and Technology Inc., Athens, GA, USA). Inhibitors of cathepsin B (CA-074 Me; 1 µM) and cathepsin K (inhibitor II; 10 µM) were from Calbiochem (San Diego, CA, US) and preincubated for 16 h. Lysosomal acidity was neutralized by NH₄Cl (10 mM, 30 min, Sigma). FAP-α activity was blocked by addition of Gly-PhP(OPh)₂ (H₂N-Gly-Pro diphenylphosphonate; 100 µM; Gilmore et al., 2006). In selected experiments recombinant TGF-β1 (10 ng/ml, Sigma) was added.

Reconstructed skin and ex vivo skin

Fibroblasts, keratinocytes and melanocytes from the same donors were isolated (Larsson et al., 2005). Skin constructs consisting of a fibroblast/collagen matrix in the bottom with a layer of melanocytes and keratinocytes allowed to form an epidermis-like layer with cornified keratinocytes covering the construct were prepared according to Li et al., 2011. Ex vivo skin (biopsies of 4 mm diameter) was obtained from excess skin from reduction plastic surgery of breast. The biopsies were placed in inserts with reconstructed skin medium II (Li et al., 2011). After exposure to UV, the samples were fixed, embedded in paraffin and subjected to immunohistochemical staining according to standard
methods using a monoclonal anti-mouse primary antibody FAP-α (1:100, sc65398, Santa Cruz Biotechnology) and polyclonal anti-rabbit primary antibody tyrosinase (1:50, ab175997, Abcam).

**Whole human genome microarray analysis**

Microarray analysis was performed in melanocytes from four different donors as described in Orfanidis *et al*, (2016). In short, young and senescent cell cultures were sampled 3 and 30 days after seeding, respectively. After isolation of total RNA, labelling and hybridisation were performed with the Affymetrix Human Genome Microarray (WT GeneTitan ST1.1; Plate type, HuGene-1_1-st-v1-16; Array type, HuGene-1_1-st-v1; Affymetrix Inc., CA, USA) and analysed at BEA (Karolinska Institutet, Stockholm, Sweden). The raw .CEL files were processed using the Agilent GeneSpring GX 13 software package (Agilent Technologies). For the gene-level experiment, the data were normalized using a quantile algorithm (Bolstad *et al*, 2003). The data is deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/); accession number GSE83922. Hierarchical clustering and heat map was determined in R (version 3.3.1) using Euclidean similarity measure with Wards linkage rule. GSE3189 identifier was used to import microarray data from a public data set (Talantov *et al*, 2005). The intersection of the common differentially expressed genes (adjusted p-value of 0.05 and minimum fold change of 1.5) from the above data sets (melanoma versus benign nevi and young versus senescent melanocytes) was used to identify lysosome associated genes that are up/downregulated in young melanocytes and melanomas. The gene set of interest (including cathepsins, TGF-β1, FAP-α, lysosome associated genes) was used in the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems) to generate the network of direct relationships between molecules.

**Gene silencing**

Depletion of FAP-α was performed with two siRNA sequences (SI00064050; SI00064057, 1 µg of each) and 6 µl RNAiFect (all from Qiagen, Germantown, MD, USA). Optimal transfection conditions
were determined by Alexa Fluor 555 labeled non-silencing siRNA, with a scrambled sequence, which was also used as negative control. siRNA targeting Lamin A/C (SI03650332) served as positive control.

**UV radiation**

The UVB source was Philips TL20W/12 tubes emitting in the spectral range 280-370 nm with a main output between 305-320 nm. For UVA, a Medisun 2000-L tube (Dr Gröbel UV-Elektronik GmbH, Ettlingen, Germany; 340-400 nm) with Schott WG 305 cut off filter (Mainz, Germany) was used. Exposure was performed in phosphate buffered saline (w/o NaHCO₃). Sublethal radiation doses of UVA 6 J/cm² (80 mW/cm²) and UVB 60 mJ/cm² (1.44 mW/cm²) were used (Larsson et al, 2005). Non-irradiated controls (sham) were analysed in parallel.

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde for 20 min at 4°C, permeabilised in PBS (without Ca²⁺/Mg²⁺) containing 0.1% saponin and 5% fetal bovine serum (Appelqvist et al, 2011). The primary antibodies used were monoclonal anti-mouse FAP-α (1:50, Santa Cruz Biotechnology) and lactate dehydrogenase (LDH, 1:100, ab52488, Abcam) followed by a secondary Alexa Fluor-488 or -594 conjugated antibody (Molecular Probes, Eugene, OR, USA). FAP-α positivity was quantified in 200 cells from randomly selected areas using a confocal microscope (LSM700, Zeiss, Heidelberg, Germany). Negative controls incubated without primary antibody did not stain.

**Western blot analysis**

Cell cultures were prepared for immunoblot as previously described (Appelqvist et al, 2011). Immunodetection was performed with monoclonal primary antibodies: FAP-α (1:1000, Santa Cruz Biotechnology), and TGF-β1 (1:1000, sc52893; Santa Cruz Biotechnology) followed by HRP conjugated corresponding secondary antibodies. GAPDH (1:50000, NB300-328, Novus Biologicals) was used as loading control and densitometric quantification was performed with Gel-Pro Analyzer 3.1 (MediaCybernetics).
To analyse proteins secreted into the medium, supernatants were collected and concentrated using Amicon Ultra-4 Centrifugal filter (5 kDa; Millipore Corporation, Billerica, MA, USA) at 4000 x g for 30 minutes at 4°C.

**Cell invasion assay**
Cells (5 x 10³/well) were cultured in the top chamber of Cultrex® 96-well basement membrane extract (BME) Invasion Assay, coated with 0.5 % BME solution consisting of laminin, collagen IV, entactin, and heparin sulfate proteoglycan. Conditioned medium from UVR exposed cells was obtained by collecting the PBS from irradiated cultures and diluting it 1:1 with 2x culture medium. Invaded cells were dissociated, treated with Calcein-AM and the fluorescence was analysed at λ<sub>ex</sub> 485/λ<sub>em</sub> 520 nm. Data were correlated to standard curves. In order to identify different cell types in the co-culture setup, fibroblasts and melanoma cells were pre-stained with Celltracker Green CMFDA (λ<sub>ex</sub> 490/λ<sub>em</sub> 520 nm) or Celltracker Red CMTPX (λ<sub>ex</sub> 580/λ<sub>em</sub> 600 nm) supplemented in medium (5 or 10 mol/L, respectively; Invitrogen Molecular Probes) at 37°C for 45 minutes.

**Migration assay**
Confluent cultures were scratched using a p200 pipet tip (Liang et al, 2007). Debris were removed by washing the cells with PBS and fresh culture medium was added. Closure of the scratch was followed during 24 h and images captured and analysed.

**Xenograft tumour model**
Zebrafish embryos, 2 days post-fertilization, were anesthetized and dechorionated. Melanoma cells, prelabeled with DiI dye, were microinjected (approx. 100-500 cells per embryo) in the perivitelline space. Embryos with fluorescent cells outside the perivitelline space were discarded. The embryos were irradiated with (UVA 20 J/cm², UVB 200 mJ/cm²) and incubated at 28°C for 3 days in the presence of anti-FAP-α (1:50, Santa Cruz Biotechnology) when indicated. The metastatic ability was assessed by quantification of distally disseminated tumour cells. Bright field and fluorescent images were captured.
**Nascent protein assay**

Cells were pre-incubated in methionine- and serum-free media for 60 min and then incubated with 25 µM Click-iT AHA (L-azidohomoalaine, C10102, Molecular Probes) with or without addition of recombinant TGF-β1 (10 ng/ml) for 4 h, 37°C. After cell lysis and sonication, the samples were centrifuged 15000 x g for 5 min, 4°C, followed by Click-IT ligation using Biotin conjugate and precipitation according to the manufacturer’s protocol (B10184, B10185, Molecular Probes). The samples were further processed for immunoprecipitation of biotin using Pierce Protein Streptavidin beads (20357, Thermo Scientific) according to Pierce™ Classic IP Kit manual (26146, Thermo Scientific).

**Subcellular fractionation**

Cells were lysed in subcellular fractionation buffer (10 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1mM EGTA, 1M DTT, PI cocktail (III), Hepes pH 7.4) on ice and passed through a 27-gauge needle, followed by centrifugation at 720 x g for 5 min to pellet nuclei. The supernatant was centrifuged 10 000 x g, 4°C for 5 min to pellet mitochondria, which was discarded, and the supernatant was further centrifuged at 100 000 x g, 4°C for 1 h, to collect the cytosol. The remaining pellet (containing the membrane fraction) was passed through a 25-gauge needle and re-centrifuged at 100 000 x g, 4°C for 1 h, followed by resuspension in fractionation buffer. The cytosolic fraction was concentrated using Amicon Ultra 3K, 4000 x g, 4°C, 40 min.

**Statistics**

Statistical evaluations were performed with one-way or two-way ANOVA, followed by Dunnett’s or Tukey’s multiple comparison post-test, respectively, for comparisons between groups using GraphPad Prism 6 Software (Version 6.05). All p-values below 0.05 were considered significant.
Results

*FAP-α is induced by UV radiation and down-regulated during senescence in human melanocytes.*

In reconstructed skin, both UVA and UVB clearly induced FAP-α expression in melanocytes (tyrosinase positive cells) whereas the surrounding keratinocytes were unaffected (Figures 1A). In line, *ex vivo* skin shows UV-induced FAP-α expression. Noteworthy, in *ex vivo* skin, the radiation-induced increase of FAP-α declined and was back at control levels after 24 h (Figure 1B). In monolayer cultures of human melanocytes, essentially, no FAP-α was located on the plasma membrane of non-irradiated cells (Figure 1C). Four hours after UVA or UVB radiation, the protein level of FAP-α was increased (Figure 1D) and located at the plasma membrane (Figure 1C). By subcellular fractionations the location of FAP-α was confirmed to the membrane fraction (Figure 1E). However, no soluble form or release of FAP-α was detected in the supernatants of non-irradiated or irradiated melanocytes (Figure 1D). As presented in Figure 1F, the radiation-induced expression of FAP-α declined time dependently, which is in line with the results from *ex vivo* skin (Figure 1B).

Previously, we have established a model showing that the gene expression in growth-arrested senescent melanocytes resembles gene expression in nevi. Using this model we were able to identify gene expression that marks a transition toward a malignant state (Orfanidis *et al*, 2016). Gene expression profiling of four matched young and senescent melanocytes, indicated 351 differentially expressed genes (minimum fold change of 2 and an adjusted p-value ≤0.01) including down regulation of FAP-α (fold change 2.5 and adjusted p-value<0.01), in the senescent cultures (Figure 2A). The senescent state was confirmed by flattened cell morphology and positive β-galactosidase staining in senescent compared to young melanocytes (Figure 2B). Comparison of the FAP-α protein expression of young and senescent cultures confirmed decreased levels of FAP-α in the senescent cultures (Figure 2C).
**Bioinformatic comparison of gene expression in nevi and malignant melanoma**

Using data from a previously published data set of gene expression from 45 primary melanomas and 18 benign skin nevi (Talantov et al, 2005), we found up-regulated expression of FAP-α (fold change; 2.8, p=1.48E-06, adjusted p-value=8.85E-06), cathepsin B (fold change; 5.9, p=5.71E-23, adjusted p-value=9.36E-20), cathepsin D (fold change; 2.2, p=9.88E-06, adjusted p-value=4.96E-05), and TGF-β1 (fold change; 1.6, p=0.003, adjusted p-value=0.007) in melanoma as compared to nevi. The intersection of the common differentially expressed genes (adjusted p-value of 0.05 and minimal fold change 1.5) from the above data sets (melanoma versus benign nevi and young versus senescent melanocytes) was used to identify lysosome-associated genes that are commonly up- or down-regulated in young melanocytes and melanomas. Applying IPA software of our gene set of interest generated a network of relationships between FAP, TGFB1 and cathepsins. The network category “post-translational modifications, protein degradation, protein synthesis” showed TGFB1 as one of the most interconnected genes (Figure 2D).

**TGF-β1 promotes cell migration and invasion through FAP-α expression**

To identify TGF-β1 producing cell types, conditioned media was collected and immunoblotted. UVA and UVB stimulated extracellular release of TGF-β1 from both young and growth arrested melanocyte cultures, whereas TGF-β1 levels were below detection limits in the respective controls. In melanoma cell lines, we found spontaneous production from control cultures that was enhanced after UVR (Table 1). However, no TGF-β1 was released from human keratinocytes or fibroblasts either spontaneously or after radiation (Table 1). To study the relationship between TGF-β1 and FAP-α expression, conditioned media from four different radiated primary melanoma cell lines was added to the corresponding non-irradiated cultures. Such treatment stimulated the expression of FAP-α (Figure 3A). By adding anti-TGF-β1 antibodies, it was demonstrated that the increase was TGF-β1 dependent (Figure 3A). Supplementation of recombinant TGF-β1 to non-irradiated cultures also increased FAP-α expression (Figure S1A, B). We found that the rise in FAP-α level was due to TGF-β1-controlled de novo synthesis of the protein (Figure S1C). In a similar manner, conditioned media from
irradiated melanoma cultures added to fibroblasts stimulated TGF-β1 dependent FAP-α expression in these cultures (Figure 3B).

Next we assessed the effect of conditioned media from radiated cultures on cell migration and invasion. Melanoma cell lines, as well as fibroblasts, were found to enhance cell growth and migration upon addition of conditioned media (Figures 3C and D). The effect was reduced by inhibition of TGF-β1. Invasion through BME coated inserts was also stimulated in a TGF-β1-dependent fashion when melanoma cultures were exposed to conditioned media from irradiated cultures (Figure 3E). Accordingly, the invasive capacity of fibroblasts cultured in conditioned media was increased (Figure 3F). These results were confirmed by addition of recombinant TGF-β1, which augmented migration and invasion in melanoma cell lines as well as in fibroblasts (Figures 2A-D). In order to investigate if senescent melanocytes could elude the growth arrested state by UVR, senescent melanocytes cultures were irradiated. No significant effect on cell migration or invasion was detected after 24 h or after 1 week (Figure S3A-D). However, increased migration and invasion was detected 1 week after addition of recombinant TGF-β1 implying its ability to break senescence in the model system (Figure S3).

Finally, we compared the time dependence for invasion in co-cultures of fibroblasts and melanoma cells. To distinguish between the two cell types, fibroblasts and melanoma cells were pre-stained with Celltracker Green and Celltracker Red, respectively. The fibroblasts launched the invasion and penetrated BME several hours before the melanoma cells (Figure 3G) and cell invasion was significantly stimulated following both UVA and UVB radiation (Figure 3G).

**TGF-β1 secretion is controlled by cathepsin activity**

To assess if release and activation of TGF-β1 is cathepsin dependent, we inhibited the cysteine cathepsins B and K using Ca-074 Me and Calbiochem inhibitor II, respectively, and inhibited the aspartic cathepsin D using anti-cathepsin D antibodies. Cathepsin inhibition caused reduced release of TGF-β1 from all four primary melanoma cell lines tested (Figure 4A). Altering the lysosomal
function by increasing the lysosomal pH using NH$_4$Cl also diminished TGF-β1 release (Figure S2E). Moreover, conditioned media from melanoma cells, pretreated with cathepsin inhibitors and irradiated before added to fibroblasts, caused diminished invasion as compared to controls (Figure 4B). Thus, cathepsins participate in the activation and release of TGF-β1.

**FAP-α promotes cell migration and invasion**

To establish that FAP-α is the primary actor during TGF-β1 stimulated invasion, we depleted FAP-α expression in melanoma cells using siRNA (Figure 4C). Conditioned media from irradiated cultures (not treated with siRNA) were added to the siRNA treated cultures, which resulted in decreased invasion of FAP-α depleted cultures (Figure 4D). In line, the invasion decreased when inhibiting FAP-α with Gly-PhP(OPh)$_2$ or anti-FAP-α (Figure S4A). The corresponding experiment performed in fibroblasts from four different individuals showed equivalent results (Figure 4E, F and S4B). Finally, to establish the importance of UVR in invasion mediated by FAP-α, we performed invasion experiments using Zebra fish embryos in a xenograft tumour model. Primary melanoma cells (FM55P) were injected in the perivitelline space and following UV radiation, the melanoma cells were allowed to invade for 48 h with or without addition of anti-FAP-α. UVR significantly increased the number of disseminated melanoma cells to the posterior part in a FAP-α dependent manner (Figure 4G and H). Taken together, our results show that cathepsins are upstream the activation of TGF-β1 in malignant melanoma cell lines. TGF-β1 is a central factor responsible for the increase of FAP-α protein level both by signaling in an autocrine manner to melanoma cells and a paracrine manner to fibroblasts. FAP-α expression has great impact on melanoma invasiveness.
Discussion

The initial steps of metastasis involves invasion through neighboring tissues, which in turn requires the degradation of the ECM and the basal membrane. In this study, the impact of UVR for increased melanoma dissemination was investigated. We present an axis of cathepsin activity, release of TGF-β1 and the expression of FAP-α, responsible for UVR stimulated cell migration and invasion. The expression and subcellular localization of cathepsins changes during cancer progression and they have been shown to be causally involved in various aspects of tumourigenesis including ECM remodeling and metastasis (Fonovic & Turk, 2014; Olson & Joyce, 2015). Moreover, cathepsin overexpression is often associated with tumour aggressiveness and poor prognosis (Palermo & Joyce, 2008; Vasiljeva et al, 2007). We have previously demonstrated that melanoma is associated with altered lysosomal function, cathepsin dependent invasion and proliferative stimulation (Eding et al, 2015). Herein, inhibition of cathepsins B, D or K in primary melanoma cells, caused decreased TGF-β1 release. In accordance, Yin et al, previously showed that cathepsin B could regulate TGF-β signaling in melanoma cells (Yin et al, 2012). These results indicate that the main tumour promoting function of cathepsins in the melanoma cells is not proteolysis of ECM per se, but rather activation of TFG-β1, promoting the up-regulation of FAP-α-mediated cancer cell dissemination.

Interactions between cancer cells and stromal cells are important for the development and progression of many cancers. We found that the normal fibroblasts alone did not invade BME-matrices but needed the initiation from the melanoma cells. TGF-β1 is a well-known activator of cancer associated fibroblasts (Löhr et al, 2001) and we found TGF-β1 dependent FAP-α expression as a sign of fibroblast activation in co-culture with melanoma cells and after supplementation of conditioned media from melanoma cultures. Previous studies have suggested that cancer cell growth could be inhibited by co-culture with normal fibroblasts (Alkasalias et al, 2014). Our results indicated that UVR interrupts such preventive capacity of the fibroblasts. Interestingly, it has been shown that UVR impairs the TGF-β signaling in human skin fibroblasts by reducing TGF-β receptor II gene expression, which results in decrease of type I procollagen synthesis (He et al, 2014). Such
mechanism would promote a switch in the fibroblast characteristics from synthesizing ECM components into a state where FAP-α expression mediates the degradation of ECM. Although both melanoma cells and fibroblasts expressed FAP-α, we found that fibroblasts led and facilitated the invasion of melanoma cells, which is in accordance with previous findings (Yin et al, 2012). A similar mechanism for invasion has been reported for squamous cell carcinoma (Gaggioli et al, 2007). Moreover, we have previously shown that UVR exposed melanoma cells produce several soluble factors including TGF-β1 (Wäster et al, 2011). A TGF-β-responsive cis-regulatory element was recently identified in the FAP promoter, indicating the central role of TGF-β for FAP-α expression (Tulley & Chen, 2014), and here we confirm de novo synthesis of FAP-α. Melanoma cells secrete TGF-β1 spontaneously, while melanocytes only showed secretion after UVR. Thus, release of TGF-β1 from melanocytes suggests ability to affect surrounding cells in the case of tissue damage. Accordingly, increased FAP-α expression facilitates ECM degradation in keloid scars (benign fibroproliferative reticular dermal lesions) indicating a general role for FAP-α during ECM remodeling (Dienus et al, 2010).

Although UVR is a well documented risk factor for development of malignant melanoma, very few experimental studies have addressed the relationship between UVR and induction of epigenetic changes. Nevi are growth arrested melanocytic lesions often harboring mutations in BRAF or NRAS (Ross et al, 2011). The initiation of a malignant transformation is therefore hypothesized to include the epigenetic breakage of the senescent state (Tsao et al, 2003). Recently, we showed that the gene expression in growth-arrested senescent melanocytes resembles that found in nevi and created a cell culture model system to identify gene expression that marks a transition toward a malignant state (Orfanidis et al, 2016). The ability of recombinant TGF-β1 to stimulate cell growth and invasion in senescent melanocyte cultures implies the potential involvement of the studied mechanism for evasion of senescence in nevi. When the gene expression in melanomas was compared with that of nevi, a network involving TGF-β1, FAP-α and lysosome associated genes was found using IPA. TGF-β1 was identified as the most interconnected gene. Interestingly, Tas et al, have suggested that the
serum levels of TGF-β1 have diagnostic, predictive, and possible prognostic roles in melanoma patients (Tas et al, 2014) indicating its clinical significance.

UV exposure of reconstructed skin, as well as skin ex vivo, showed FAP-α up-regulation in melanocytes indicating that the finding has physiological relevance. Consequently, sun exposure of a primary melanoma of the skin could initiate invasion and further progression of the tumour, emphasizing the necessity of research in the area. Future strategies for melanoma prevention need to focus on more efficient protection against UV-induced damage. ECM degrading enzymes including matrix metalloproteinases and cathepsins have received much attention as potential future therapeutic targets (Deryugina & Quigley, 2006). However, their applicability is limited due to their widespread expression. In contrast, targeting of FAP-α might be an attractive alternative since it is highly expressed in tumour stroma and absent in normal adult tissue (O’Brien & O’Connor, 2008). The clinical relevance of FAP-α inhibition for possible identification and treatment of advanced disease is underlined by our findings on tumour dissemination in zebrafish embryos. So far, FAP-α inhibitors have not demonstrated sufficient efficacy in clinical trials (reviewed in Jiang et al, 2016). Instead the development of better FAP-α blockers are required, and combination strategies addressing the heterogeneity of melanomas. Recently, anticancer drugs, encapsulated by cleavable amphiphilic peptide assembled into nanocarriers, were shown to disassemble upon cleavage by FAP-α, resulting in rapid and efficient release of encapsulated drugs specifically at the tumour sites (Ji et al, 2016).

In conclusion, our study indicates UVR as an initiating factor engaging an axis from cathepsins via TGF-β1 release, which results in increased protein expression of FAP-α in both malignant cells and the surrounding stroma (Figure 5). FAP-α causes ECM degradation enabling cell migration and invasion as well as tumour dissemination in zebrafish. Thus, UV exposure might be involved in the early migration and invasion process of melanoma cells emphasizing the important role of UV
protection not only to prevent melanoma development, but also to avoid migration and invasion of an established disease.
Conflicts of interest

The authors state no conflict of interest.

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**Figure 1:** UV radiation augments FAP-α expression. Samples were irradiated with UVA (6 J/cm²) or UVB (60 mJ/cm²). A) Artificial skin constructs stained for FAP-α (green) and the melanocytic marker tyrosinase (red) in combination with DAPI stained nuclei (blue) and bright field in the merged image 4 hours after UVR. B) Normal ex vivo skin stained 2 and 24 h after radiation showing representative merged images of FAP-α (green), the melanocytic marker tyrosinase (red) and DAPI stained nuclei (blue). C) Representative merged images of immunofluorescence of FAP-α (green), cytosolic marker protein (LDH, red) and bright field and D) immunoblotting of active and secreted FAP-α 4 hours after UVR. GAPDH is used as loading control. E) Immunoblotting of FAP-α, E-cadherin (E-cad, plasma membrane marker), and GAPDH (cytosolic marker) in subcellular fractions after radiation. F) Quantification of FAP-α positive melanocytes following radiation (mean ± SD; n=4; *p<0.05).

**Figure 2:** FAP-α is downregulated in senescence and found in a TGFB1 centered gene network.
Human melanocytes from 4 donors were cultured for 3 days (young) and until they stopped dividing and displayed senescent morphology (minimum of 30 days). Gene expression was analysed using Affymetrix microarray. A) Heat map presenting hierarchical clustering results (using Euclidean similarity measure with Wards linkage rule) from microarray expression profile data and the expression of 351 selected genes. Gene expression ratios are displayed by applying progressively brighter shades of red (up-regulated) or green (down-regulated). B) Staining of β-galactosidase in young and senescent melanocytes. Representative images are presented. C) Immunoblotting of FAP-α in 4 young (Y) and the corresponding senescent (S) cultures. GAPDH used as an internal loading control. D) Generated network of relationships between FAP, TGFB1 and cathepsins (CTS) using Ingenuity Pathway Analysis software. The network entitled “post-translational modifications, protein degradation, protein synthesis” show TGFB1 as the most interconnected gene.
**Figure 3:** FAP-α expression is regulated by TGF-β1 and stimulates cell migration and invasion.

Primary melanoma cells (FM55P, WM115, WM278, WM793) were irradiated with UVA (6 J/cm²) or UVB (60 mJ/cm²) and when indicated treated with anti-TGF-β1 (200 ng/ml). Conditioned media were collected and supplemented to the respective non-irradiated primary melanoma cells (FM55P, WM115, WM278, WM793) or normal skin fibroblasts (FB1-4). Quantification of FAP-α expression in A) primary melanoma cells and B) fibroblasts (n=4; * p<0.05). Estimation of migration in C) primary melanoma and D) fibroblast. The results are presented as the percentage of cells that invaded the scratch during 24 h (n=4; * p<0.05). Invasion of E) primary melanoma and F) fibroblasts using Cultrex BME. The results are presented as the percentage of invading cells relative to the total number of cells (n=4; * p<0.05). G) Time dependent invasion of co-cultures of primary melanoma cells (labelled with Celltracker Red CMTPX) and fibroblasts (labelled with Celltracker Green CMFDA) (n=4). * p<0.05 compared to time 0 h.

**Figure 4:** Cathepsins act upstream of TGF-β1 and UVR stimulates FAP-α dependent invasion. A) and B) Primary melanoma cells (FM55P, WM115, WM278, WM793) were irradiated with UVA (6 J/cm²) or UVB (60 mJ/cm²) and when indicated treated with inhibitors of cathepsin B (CA-074 Me; 1 µM), anti-cathepsin D (anti-cat D; 42 µg/ml), cathepsin K (Inh II; 10 µM). The conditioned media were collected. A) TGF-β1 release to the medium was analysed by immunoblot. Samples were collected from equal number of cells and representative blots are shown (n=4). B) Invasion of fibroblasts (FB1-4) through Cultrex BME after supplementation with the conditioned media from melanoma cultures. C and D) FAP-α was depleted in primary melanomas (WM115, WM793, WM278 and FM55P) with two different siRNA sequences (S1 and S2; 1 µg of each). C) Immunoblotting of FAP-α. One representative blot out of four is shown. D) Invasion assay. E and F) FAP-α was depleted in human skin fibroblasts (FB1-4) with two different siRNA sequences (S1 and S2; 1 µg of each). E) Immunoblotting of FAP-α. One representative blot out of four is shown. F) Invasion assay. G and H) Invasion assay in Zebra fish embryos injected with primary melanoma cells (FM55P; 100-500 cells, labeled *in vitro* with Dil dye) in
the perivitelline space and when indicated anti-FAP-α (20.6 µg/ml) was added. After microinjection
the embryos were irradiated with UVA (20 J/cm²) or UVB (200 mJ/cm²) and the distally posterior
disseminated tumour cells were quantified after 3 days. G) Bright field and fluorescent images
illustrating tumour cell dissemination. H) Quantification of distally posterior fluorescent cells. Values
shown are mean± SD of 3 independent experiments.

**Figure 5: Intercellular signaling between melanoma cells and stromal fibroblasts.** UV initiates an
axis from cathepsins that stimulates release and activation of TGF-β1. TGF-β1 transactivates the FAP-
α gene and increase the expression of FAP-α, which causes ECM degradation enabling cell migration
and invasion.
Supplementary Figure Legends

**Figure S1. Recombinant TGF-β1 stimulates FAP-α production.** Primary melanoma cells (FM55P, WM115, WM278, and WM793) and human fibroblasts (FB1-4) were treated with recombinant TGF-β1 (10 ng/ml). Quantification of FAP-α expression in A) primary melanoma cells and B) fibroblasts. C) Immunoblotting of nascent biotinylated proteins and FAP-α after Click-IT AHA ligation in combination with immunoprecipitation in melanocytes supplemented with/without (+/-) supplementation of recombinant TGF-β1 (4 h, 10 ng/ml).

**Figure S2. Recombinant TGF-β1 stimulates FAP-α expression migration and invasion.** Primary melanoma cells (FM55P, WM115, WM278, and WM793) and human fibroblasts (FB1-4) were treated with recombinant TGF-β1 (10 ng/ml). Migration analysis of A) primary melanoma and B) fibroblasts. The results are presented as the percentage of cells that invaded the scratch during 8 h (n=4; * p<0.05). Invasion of C) primary melanoma and D) fibroblasts using Cultrex BME. The results are presented as the percentage of invading cells relative to the total number of cells. E) Conditioned media from melanoma cell lines were collected and immunoblotted for detection of TGF-β1 after NH₄Cl (10 mM, 30 min) supplementation. One representative blot out of 4 is shown.

**Figure S3. Senescent melanocytes becomes activated after UVR.** Human melanocytes from 3 donors were cultured until they stopped dividing and displayed senescent morphology (minimum of 30 days). Senescent melanocytes were then irradiated with UVA (6 J/cm²), UVB (60 mJ/cm²) or treated with recombinant TGF-β1 (10 ng/ml). Migration analysis of senescent melanocytes presented as the percentage of cells that invaded the scratch during A) 24 h or B) 1 week. Invasion of senescent melanocytes using Cultrex BME presented as the percentage of invading cells relative to the total number of cells C) 24 h and D) 1 week after treatment. (n=3; * p<0.05).
Figure S4. Inhibition of FAP-α by anti-FAP-α and Gly-PhP(OPh)₂ prevents invasion. Primary melanoma cells (FM55P, WM115, WM278, and WM793) and human fibroblasts (FB1-4) were irradiated with UVA (6 J/cm²) or UVB (60 mJ/cm²) and when indicated treated with anti-FAP-α (20.6 µg/ml) and Gly-PhP(OPh)₂ (100 µM). Invasion of A) primary melanoma and B) fibroblasts using Cultrex BME. The results are presented as the percentage of invading cells relative to the total number of cells. (n=4, *p<0.05).
Figure 1. Wäster et al., 2017
Figure 2. Wäster et al., 2017
Figure 3. Wäster et al., 2017
Ultrasound irradiation

Cell-extracellular matrix interactions

Fibroblast

Intercellular communication

Melanoma cells

Basement membrane

FAP-α
Cathepsin B/D
TGF-β1
TGF-receptor

Figure 5. Wäster et al., 2017
Table 1. TGF-β1 secretion. Data derived from immunoblotting (optical density compared to local background, mean ± SD, n=4).

<table>
<thead>
<tr>
<th>Cell type:</th>
<th>TGF-β1 (optical density)</th>
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<tr>
<td>Control</td>
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<tr>
<td>UVB</td>
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<td>Primary melanomas</td>
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Figure S1. Wäster et al., 2017
Figure S2. Wäster et al., 2017
Figure S3. Wäster et al., 2017
Figure S4. Wäster et al., 2017