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Aberrant expression of kallikrein-related peptidase 7 is correlated to human melanoma aggressiveness by stimulating cell migration and invasion

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Abstract

Members of the tissue kallikrein-related peptidase (KLK) family not only regulate several important physiological functions, but aberrant expression has also been associated with various malignancies. Clinically, KLKs have been suggested as promising biomarkers for diagnosis and prognosis in many types of cancer. As of yet, expression of KLKs and their role in skin cancers are, however, poorly addressed. Malignant melanoma is an aggressive disease associated with poor prognosis. Hence, diagnostic biomarkers to monitor melanoma progression are needed. Herein, we demonstrate that although mRNAs of several KLKs are aberrantly expressed in melanoma cell lines, only the KLK7 protein is highly secreted *in vitro*. In line with these findings, ectopic expression of KLK7 in human melanomas and its absence in benign nevi was demonstrated by immunohistochemistry *in vivo*. Interestingly, overexpression of KLK7 induced a significant reduction in melanoma cell proliferation and colony formation. Moreover, KLK7 overexpression triggered an increase in cell motility and invasion associated with decreased expression of E-cadherin and an up-regulation of MCAM/CD146. Our results demonstrate, for the first time, that aberrant KLK7 expression leads to a switch from proliferative to invasive phenotype suggesting a potential role of KLK7 in melanoma progression. Thus, we hypothesize that KLK7 may represent a potential biomarker for melanoma progression.

Abbreviations:

ELISA : enzyme-linked immunosorbent assay

EMT : epithelial–mesenchymal transition

ERK1/2 : Extracellular signal-Regulated Kinases 1 & 2

FCS *:* Fetal Calf Serum

GAPDH : Glyceraldehyde-3-Phosphate Dehydrogenase

IHC/ISH : Immunohistochemistry./situ hybridization

IL-1-β : Interleukin-1

KLK : kallikrein-related peptidase

MAP-Kinase : Mitogen-activated protein kinase

MCAM : melanoma cell adhesion molecule

MM : metastatic melanoma

MMP9 : Matrix metallopeptidase 9

NHEK : Normal Human Epidermal Keratinocytes

NHEM : normal human epidermal melanocytes

PARs : protease-activated receptors

PCR : Polymerase Chain Reaction

PM : primary melanoma

qPCR : quantitative polymerase chain reaction

RT-PCR *:* Reverse Transcription PCR

TGF-β *:* Transforming growth factor beta

uPAR :plasminogen activator receptor

1. Introduction

Despite recent advances in the understanding of oncogenic mechanisms and therapeutic intervention, melanoma is the most aggressive skin cancer with poor prognosis in the metastatic stage. The development of melanoma begins with the malignant transformation of normal human epidermal melanocytes (NHEM) located within the basement membrane of the skin. Clinical and histopathological evidence suggests that melanoma develops sequentially, progressing from primary *in situ* melanomas, to invasive primary lesions, and finally to metastases (Haass and Herlyn, 2005). The outlined steps involve molecular changes that include acquisition of the epithelial–mesenchymal-like transition (EMT-like) associated to changes in cell surface adhesion molecules and activation of signaling pathways finally leading to cell dissemination (Haass and Herlyn, 2005). Despite extensive efforts concerning characterization of malignant melanoma, no specific molecular markers are currently available that are clearly related to the progression of this disease. In addition, it has been suggested that treatment failure is due to the heterogeneity of melanoma cells, which might be driven by microenvironmental factors (Postovit et al., 2006). Proteases are promising candidates for such molecular markers since they have been shown to be involved in invasion of cancer cells and metastasis due to their ability to degrade extracellular-matrix components. Moreover, a growing body of evidence has identified diverse mechanisms by which proteases affect cancer progression and metastasis through complex processes that involve cleavage of cell adhesion molecules, growth factors, and cytokines (Sevenich and Joyce, 2014). Besides some other serine proteases, certain members of the kallikrein-related peptidase (KLK) family serve as signaling molecules controlling cell functions also through specific membrane receptors, the protease-activated receptors (PARs) (Darmoul et al., 2001; Darmoul et al., 2003; Darmoul et al., 2004; Ramsay et al., 2008; Gratio et al., 2010; Gratio et al., 2011).

The KLK superfamily comprises fifteen (KLK1-KLK15) trypsin-/chymotrypsin-like serine proteases which are secreted into the extracellular space of a wide range of tissues and, furthermore, are also suggested to be involved in tumor progression (Borgono and Diamandis, 2004; Emami and Diamandis, 2007; Kryza et al., 2016). Several KLK members have been shown to be concomitantly upregulated in many cancers depicting them as valuable biomarkers to distinguish between normal and cancerous phenotype, but also to predict the course of cancer disease and response to cancer therapeutics of patients (Yousef et al., 2003; Kontos and Scorilas, 2012).

One of the most intensively studied tissues for evaluation of KLK functions is the skin. In normal skin, among the multiple KLKs detected, KLK7 (chymotrypsin-like), KLK5 and KLK14 (both trypsin-like) are considered as the major proteases (Fischer and Meyer-Hoffert, 2013; Kalinska et al., 2016). They have been proposed to function as desquamatory enzymes by causing proteolysis of intercellular cohesive structures in the stratum corneum (Caubet, 2004). Elevated expression of KLKs - in particular KLK5 and KLK7 - have in fact been found in skin diseases involving skin barrier disorders (Fischer and Meyer-Hoffert, 2013; Kalinska et al., 2016).

During tumor progression, higher levels of KLK7 in tumor tissue have been mainly associated with poor prognosis in a variety of cancer types, even when the expression level was found to be down-regulated in relation to the corresponding non-malignant, normal tissue (Devetzi et al., 2013; Stefanini et al., 2015; Kryza et al., 2016). Elevated KLK7 expression is, for example, correlated with short survival in colon, ovarian, and pancreatic cancer (Devetzi et al., 2013; Dorn et al., 2014; Iakovlev et al., 2012). So far, analysis of KLK expression levels and functions in skin cancer has not been addressed in detail. Still, using microarray technology approaches, gene network analysis and immunohistochemistry, recent studies point to the importance of KLKs in melanoma progression. Indeed, it has been shown that KLK6, 7, 8, and 13 are co-ordinately expressed in melanoma progression. In addition, KLK7 was found to be associated with good prognosis and survival outcome of patients with primary melanoma (Martins et al., 2011; Rezze et al., 2011). Concerning KLK6 , a controversial study reported that this protein is not expressed by melanoma cells but rather by keratinocytes and stromal cells of the microenvironment (Krenzer et al., 2011).

In the present study, we demonstrate, for the first time, aberrant expression and secretion of multiple KLKs in melanoma cell lines. KLK7 displayed ectopic expression in melanoma cells *in vitro* and in resected tumors from patients with primary and metastatic melanomas but was absent in nevi. Furthermore, we clearly show that KLK7 overexpression in melanoma cells induces a decrease in cell proliferation and colony formation. Concurrently, a loss of E-cadherin expression and upregulation of MCAM/CD146 is observed, which is associated with an increase of cell motility and cell invasion. Thus, these data suggest that KLK7 is not only a potential biomarker for melanoma progression, but also play a role in tumor invasion.

Materials and Methods

2.1 Reagents

Neomycin (or G418), DMEM, RPMI 1640, and HAM's F12 medium were purchased from Life Techonologies (Cergy-Pontoise, France), the Nucleospin RNA kit from Macherey–Nagel (Düren, Germany). Antibodies were purchased from the following vendors; human KLK7 polyclonal antibody (#GTX103548) from GeneTex Inc. (Irvine, CA); E-cadherin (32A8) (#5296) and mouse phospho-specific antibodies to ERK1/2 (Thr202/Tyr204) (#9106) from Cell Signaling Technologies (Beverly, MA); polyclonal anti-ERK1/2 (#SC-94) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); MCAM/CD146 from R&D systems (Lille, France); Peroxidase-conjugated anti-mouse (#115-035-068) and anti-rabbit (#111-035-144) antibodies were from Jackson ImmunoResearch (West Grove, PA). Alexa Fluor® 488 anti-mouse IgG from Invitrogen (Carlsbad, CA). Purified rabbit IgG were obtained from Sigma-Aldrich (Lyon, France).

2.2 Cell culture

Human melanoma cell lines (Colo 792, MeWo, 501Mel, A-375, Colo 794, Colo 829, Dauv-1, M74, MM170, SK-Mel5, SK-Mel28, WM115, WM 266-4, C8161, HM11, SBcl2, WM1361, WM852, XP44RO, MM127, MT10, M230 and WM1791C) were kindly provided by Nicolas Dumaz (INSRM U976, France) (Dumaz et al., 2006). All melanoma cell lines have been genotyped to verify their authenticity. WM115 is derived from a primary melanoma, and WM266.4 a metastatic melanoma cell line isolated from the same patient. Cells were cultured in high glucose DMEM or RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2. Normal neonatal human epidermal melanocytes (NHEM) were from PromoCell (Heidelberg, Germany) and Cascade Biologics (Nottinghamshire, UK). They were grown in KBM-Gold medium supplemented with Stem Cell Factor (SCF) (Lonza, Aubergenville, France) or in medium 154 supplemented with human melanocyte growth supplement (Cascade Biologics). HaCaT, immortalized keratinocytes, were cultured in DMEM supplemented with 10% FBS are from ATCC (Rockville, MD).

2.3 Reverse transcription polymerase chain reaction (RT-PCR)

Four micrograms of total RNA were reverse-transcribed using oligo (dT) primers and Thermoscript kit (Thermofisher Scientific) according to the manufacturer's instructions. Ten percent of the reaction was used as template in the PCR reaction to amplify human KLK4, KLK5, KLK6, KLK7, KLK8, KLK10 or

KLK14. Primer sequences and conditions are described in Table 1S (**Supplementary Table 1**). GAPDH cDNA amplification was used as an internal control. After 35 cycles of amplification, PCR products were identified by electrophoresis in 2% agarose gels followed by SYBR$^{®}$Safe staining (Invitrogen, Carlsbad, CA).

2.4 Quantitative reverse transcription PCR

One µg of total RNA was reverse transcribed and PCR reactions were then performed in duplicate using the Power SYBR$^{®}$ Green PCR Master Mix kit (Applied Biosystems) following the manufacturer's instructions. The final 10 µL reaction volume included 5 µL of 2 × Master Mix, 50 nM of each primer and 2 µL cDNA. The following primers were used: 5'CCCAGTGCTCTGAATGTCAA3' (forward) and 5'AGTGGGAATCTCGTTCATCC3' (reverse) for KLK7; 5'TGGGTGTGAACCATGAG-AAGTATG3' (forward) and 5'GGTGCAGGAGGCATTGCT3' (reverse) for GADPH, a housekeeping gene used as an internal standard. The QPCR conditions were as follows: 10 min at 95 °C, then 40 cycles of amplification at 95 °C for 15 s and 60 s at 60 °C. Relative quantification of the target gene expression was done using the comparative cycle threshold (C_t) method (Sequence Detection Systems 2.0; Applied Biosystems). C_t was normalized to GAPDH ($\Delta C_t = C_t$ sample$-C_t$ GAPDH).

2.5 KLK ELISA assays

Melanoma cells were seeded at 40,000 cells/well in 12 well plates. At confluence, the conditioned medium was collected for antigen determination of various KLKs using a non-competitive immunoassay as previously described (Shaw and Diamandis, 2007) (Gratio et al., 2011). whereas the cells were detached and counted.

2.6 Tissue immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue samples (n = 38) encompassing 6 cases with nevi, 18 cases with primary melanoma and 14 cases with metastasis (Pathology Department of Bichat-Claude Bernard Hospital, Paris). Tissues were analyzed in accordance with the requirement of the Human Research Committee of the Bichat-Claude Bernard Hospital and according to the Declaration of Helsinki as adopted by the French Bioethical Law (2004). Tumors were staged according to the 7th International Union Against Cancer (UICC). KLK7 immunostaining was performed using a Leica Bond Max Automated IHC/ISH Stainer and the Bond Polymer Refine Detection Kit (Leica Microsystems Inc, Nanterre, France) according the manufacturer's instructions. Briefly, the machine performs all steps of deparaffinization, heat-induced antigen retrieval in high-pH (pH, 9) Bond Retrieval Solution at 100°C. After endogenous peroxidase quenching, anti-human KLK7 (N1C3) polyclonal antibody or rabbit IgG control was applied both at a 1:100 dilution. After incubation, Post Primary Antibody, and thereafter, Poly-HRP-IgG reagent was added. Detection was performed using DAB (3,3'-diaminobenzidine tetrahydrochloride) Refine. Nuclei were counterstained with Mayer’s hemalum solution. Specificity of KLK7 immunostaining has been demonstrated by using rabbit IgG (data not shown) and by omitting the primary antibody (Walker et al., 2014). Staining was assessed by three independent observers employing a semi-quantitative methodology: first, the percentage of KLK7 immunostained cancer cells was evaluated and second, the staining intensity was scored on a scale between no (0), weak (+1), moderate (+2), and strong (+3) staining.

2.7 Western blot analysis

Recombinant human proKLK7 was produced and purified from yeast cells as described earlier (Stefansson et al., 2008). The enzyme was then activated using thermolysin and the activity was routinely tested against a fluorogenic substate (data not shown). Quiescent cells were treated with recombinant active recombinant KLK7 at different concentrations and for various time periods as indicated in the Results section. Cells were lysed with RIPA buffer as described (Darmoul *et al*. 2004). Equal amounts of extracts (25 µg) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were incubated in blocking buffer (20 mM Tris, 50 mM NaCl) containing 5% (w/v) low-fat milk and 0.1% (v/v) Tween 20 and then probed with a monoclonal phospho-specific antibody directed to ERK1/2 (1:2,000) overnight at 4°C. Membranes were stripped in Stripping Buffer (Invitrogen) and reprobed with a polyclonal anti-ERK1/2 antibody (1:1,000) that recognizes total ERK1/2 regardless of its phosphorylation state as loading controls. Proteins were revealed applying the Signal® Chemiluminescent Substrate (Thermo Scientific) on an Image-Quant imaging system.

2.8 Transfection of the KLK7 expression plasmid and selection of stable transfectants

The cDNA encompassing the coding region of KLK7 was inserted into the mammalian expression vector pRc/RSV (Invitrogen, Carlsbad, CA) yielding pRc/RSV-KLK7 as described (Prezas et al., 2006). pRc/RSV (vector control) and pRc/RSV-KLK7, respectively, were transfected into the KLK7-deficient melanoma cell line M74 (see Figure. 1), using the lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Fourty eight hours after transfection, the culture medium was replaced by selective medium containing G418 (1 mg/ml). The batch of neo-resistant pRc/RSV-KLK7-transfected M74 cells were denoted as M74-H, a selected clone, which expressed high amounts of KLK7, as M74-D6. M74 cells, stably transfected with the vector only, were designated as M74-mock cells.

The catalytic triad serine to alanine mutant-KLK7-S195A plasmid was generated by site-directed mutagenesis (**Supplementary Figure 1**). In the set of experiments, designed for analysis of the active site mutant, M74 cells transfected with KLK7 mutant (KLK7-S/A) cDNA were denoted M74-KLK7-S/A.

2.9 Cell proliferation assay

Cell proliferation was determined either by direct cell counting or using the WST-1 Cell Proliferation Assay Kit (Roche Diagnostics GmbH, Mannheim, Germany).

For direct counting, cells were seeded at a density of 2×10^4 cells (48-well plates) and incubated at 37°C for 24, 48, 72 or 96 h. Cells were detached from triplicate wells and counted in a hemacytometer. Cell death was evaluated by staining of the cells with trypan blue. At least three independent experiments were performed for each experimental condition.

For the WST-1 assay, cells were seeded at a density of 5×10^3 cells (96-well plates) and incubated at 37°C for 24, 48, 72 or 96 h. WST-1 was added to each well and the plate was incubated in 5% CO_2 at 37 °C for 2 h. Plates were finally analyzed by measuring the absorbance at 450 nm versus the reference wavelength of 630 nm using a scanning multiwell spectrophotometer. Three independent experiments were performed for each experimental condition.

2.10 Clonogenic assay

To test the ability of single cells to grow into a colony, KLK7-expressing cells (M74-D6 and M74-H) or vector-control cells (M74-mock) were plated at a low density (1,000 cells/well) in 6-well plates and allowed to generate single colonies for 14 days. The colonies were washed twice in PBS, then stained with 0.5% (v/v) crystal violet-20% methanol, imaged and quantified using an Image Quant™ LAS 4000 digital imaging system and the Image J software (GE Healthcare, Piscataway, NJ). At least three independent experiments were performed in duplicate.

2.11 Immunofluorescence staining

E-cadherin and MCAM/CD146 immunofluorescence detection was performed with cells grown on glass coverslips (IBD). Cells were washed three times in PBS, fixed in 2% paraformaldehyde, washed three times in PBS, and then incubated with PBS containing 2% BSA for 15 min prior to application of the primary anti-E-cadherin or anti-MCAM/CD146 antibodies (1:200) for 2 h at room temperature. Subsequently, cells were incubated for 45 min with the secondary antibody goat anti-mouse IgG coupled to Alexa-488 fluor. Negative controls were obtained by omitting primary antibodies. Finally, the cells were mounted in Vectashield medium containing DAPI Dye (Vector, Peterborough, UK) and examined using a fluorescence microscope (*Zeiss*, Jena, Germany).

2.12 Cell migration and Matrigel™ invasion assay

For the *in vitro* cell migration assay, 8-µm pore-size Transwell® inserts (Ibidi, Martinsried, Germany) were used according to the manufacturer's instructions. The chambers were placed into 24-well wells containing 750 µl of RPMI medium supplemented with 10% FCS as a chemoattractant. Cells (2×10^4) were added to the upper well of each chamber in 200 µl of serum-free RPMI medium.

For the cell invasion assay, Transwell® inserts were coated with 10 µg of Matrigel™ (Biocoat, BD Biosciences, San Jose, CA) in 100 µl of RPMI at 37 °C. The coated chambers were air-dried for 6 h. The chambers were then placed into 24-well dishes containing 750 µl of RPMI medium supplemented with 10% FCS. Cells (5×10^4) were seeded into the upper chamber of the device in 200 µl of serum-free RPMI medium. After a 24 h-incubation period, migrated or invaded cells on the lower side of the insert were stained with a 0.5% (v/v) crystal violet-20% methanol solution and imaged by bright-field microscopy in six random fields at magnification ×4. The number of stained cells after migration or invasion was counted with the Image-J software.

2.13 Statistical analysis

Data show the mean values of at least three independent experiments. The GraphPad Prism statistical tool was used to perform the one-way ANOVA. A p-value < 0.05 was considered statistically significant (NS > 0.05, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

3. Results

3.1 Kallikrein-related peptidases are ectopically expressed in human melanoma cell lines

Since recent studies point to the importance of KLKs in melanoma progression (Martins et al., 2011; Rezze et al., 2011), we aimed at investigating the expression pattern of KLKs in melanoma cells. mRNA levels of several KLKs were analyzed by RT-PCR in 23 human melanoma cell lines. We first focused on KLK5, KLK6, KLK7, and KLK14 that have been shown to play major physiological roles in the skin (Fischer and Meyer-Hoffert, 2013; Kalinska et al., 2016). As shown in **Figure 1**, KLK5, KLK6, KLK7 and KLK14 mRNA were expressed in 34%, 86%, 69%, and 100% of the analyzed cell lines, respectively. Also other KLKs - KLK4, 8, and 10 - were found to be present in 30%, 13%, and 39 % of melanoma cell lines, respectively (for a summary of the expression pattern of all analyzed KLKs see **Table 1**). In contrast, all *KLKs* tested were absent or only very weakly expressed in normal human melanocytes (NHEM) **(Figure 1).** As expected, keratinocyte-like HaCaT cells, taken as a control, express high levels of the *KLK* genes tested in our study (Lundwall and Brattsand, 2008). Thus, our data suggest that melanoma cell lines ectopically express certain *KLK* genes that are absent or very weakly expressed in normal melanocytes. We did not find major differences in the *KLK* expression patterns of the primary melanoma cell lines (*i.e.* WM115, Dauv1, WM1361, HM11 and SBcl2) as compared to metastatic cell lines **(Table 1** and **Supplementary Table 2)**. In addition, no correlation was found between expression of *KLKs* and BRAF, NRAS or C-Kit mutations **(Supplementary Table 2)** which are the most frequently mutated oncogenes in melanomas (Flaherty et al., 2012).

3.2 Detection of secreted KLKs in supernatants of human melanoma cells

Protein expression of various KLKs was investigated by measuring secretion of these proteins into the conditioned media from 23 human melanoma cell lines and from normal melanocytes. The concentrations of KLK4-8, KLK10, KLK13 and KLK14 in the cell line supernatants were quantified using immunoassays as previously described (Shaw and Diamandis, 2007; Gratio et al., 2011). As shown in **Figure 2,** many KLKs were detected in the supernatants. However, mRNA levels did not in all cases reflect the protein levels (compare to **Table 1**), a common phenomenon due to the complexity of the transcriptional regulation (Kryza et al., 2016). Interestingly, KLK7 was the major secreted KLK by human melanoma cell lines (**Figure 2A**). Highest KLK7 levels (958 +/- 106 ng/L/10^6 cells; ~38 pmol/L) were observed in the conditioned media from SK-Mel5 cells followed by M127, MT10, M230, WM1791c, HM11, SK-Mel28 and WM115 cells (**Figure 2B**). In accordance with the PCR results, KLK7 immunodetection in normal melanocytes (NHEM) as well as in the other analyzed cell lines was very low or below the detection limit of the current protocol. Subsequent quantitative PCR analysis confirmed that SK-Mel5, A375, MeWo, Dauv1, SK-Mel 28, WM115, and MT10 cells display the highest *KLK7* mRNA levels whereas NHEM cells, obtained from two different sources (**Supplementary Figure 2**), do not express KLK mRNA. Taken together, these data suggest that melanoma cell lines express and secrete distinct amounts of KLKs that could potentially act in an autocrine manner and play a role in melanoma progression.

3.3 KLK7 expression in primary and metastastic melanomas *in vivo*

To validate ectopic expression of KLK7 in melanoma *in vivo*, we next examined the pathological relevance of our observations by performing immunohistochemistry of KLK7 in primary and metastatic melanomas *versus* nevi. As shown in **Figure 3**, KLK7 was not or barely detected in nevi, but clearly elevated in primary and metastatic melanomas. Staining was localized to the cytoplasmic compartment of the cells. Normal appearing epidermal cell layers adjacent to nevi (**Figure 3A-a**) or to primary melanoma in (**Figure 3A-b**) showed the strongest KLK7 staining in keratinocytes, in concordance with its described high level expression in the stratum corneum layers (Egelrud et al., 2005). In both primary and metastatic melanomas, the intensity of KLK7 staining varied in the different patient samples analyzed and was evaluated as weak (**Figure 3A-b** and **c**), moderate (**Figure 3A-d**), and strong (**Figure 3A-e** and **f**), respectively. Interestingly, analysis of the intensity of KLK7 immunoreactivity revealed that metastatic melanoma tissues showed a higher average of cells with strong intensity compared to primary melanoma (13.5 % *versus* 3.5 %). The number of cells with moderate intensity was also higher in metastatic melanoma samples compared to primary melanomas (25 % *versus* 18 %), while, the average number of cells with weak intensity staining was higher in primary melanomas compared to metastatic melanoma samples (42 % *versus* 56 %) (**Figure 3B**). No specific staining was seen when the primary antibody was replaced with the rabbit IgG (not shown). These observations show that human melanoma cells aberrantly express high levels of KLK7 in contrast to benign melanocytes or benign nevi. This suggests that KLK7 expression may represent a potential marker for melanoma progression.

3.4 KLK7 enhances ERK1/2 activation in human melanoma cell lines

Because KLK7 has been proposed to act as a proliferative factor in many cancer types (Walker et al., 2014; Kryza et al., 2016), we next investigated the effect of KLK7 on MAP kinase (ERK1/2) phosphorylation, an upstream kinase in the proliferation pathways. Addition of KLK7 (10 nM) to quiescent MeWo cells, at various time points, induced a rapid and significant phosphorylation of ERK1/2, reaching a maximum within 5-10 min (**Figure 4A**). In addition, KLK7 induced ERK1/2 phosphorylation in the range between 5 nM and 20 nM, whereby maximum ERK1/2 phosphorylation was obtained already with 10 nM KLK7 (**Figure 4B**). These experiments suggest that KLK7 activates the MAP-kinase pathway in melanoma cells, possibly playing an important role in melanoma proliferation.

As members of KLKs have been suggested to modulate cytokine and TGF-beta in some cancer types (Kryza et al., 2016), we also analyzed phosphorylation of Smad 2 (TGF beta pathway), Stat3 (cytokine and growth factor pathways) in two different *in vitro* cell models; (1) in KLK7-overexpressing melanoma M74 cells (upon stable transfection with a KLK7 expression vector, see section below), grown under serum-free conditions, and (2) in Mewo cells, stimulated with recombinant KLK7 under serum-free conditions. In neither of the models, an evidence for a KLK7-mediated modulation of cytokine, growth factor or TGF-beta pathways was found **(Supplementary Figure 3)**.

3.5 KLK7 overexpression induces reduction of melanoma cell growth and colony formation

To examine the possible function of KLK7 in melanoma cells, we stably overexpressed KLK7 in the M74 melanoma cell line, using the KLK7 expression vector pRcRSV-KLK7. M74 cells were chosen because in these cells expression of KLK7 is very low **(**see **Figure 1** and **Table 1**). We tested both a selected clone (M74-D6) and batch-transfected cells (M74-H). As shown in **Figure 5A**, QPCR analysis revealed that the selected M74-D6 clone as well as the batch-transfected cells M74-H, express significant amounts of KLK7 mRNA. As expected, the vector-transfected control cells (M74-mock) did not express KLK7 mRNA. Moreover, ELISA measurements showed that in M74-D6 and M74-H cells the KLK7 protein was also secreted into the cell culture medium (**Figure 5B**). Notably, the secreted KLK7 levels in M74-H cells were comparable to those secreted by HaCaT keratinocyte-like cells (**Figure 5B, inset**).

Although previous studies have suggested that KLK7 acts as a proliferative factor in a number of cancer types (Walker et al., 2014; Stefanini et al., 2015; Xi et al., 2015; Kryza et al., 2016), in the M74-D6 and M74-H melanoma cell lines, KLK7 overexpression surprisingly suppressed cell growth compared to M74-mock cells (**Figure 6A**)**.** M74-transfected cells expressing a KLK7 mutant (KLK7-

S/A), where the catalytic serine residue is replaced by alanine (**Supplementary Figure 1**), did not show any significant differences in cell proliferation compared to vector control cells (**Figure 6B**). Similarly, in a colony formation assay both the M74-D6 clone and the batch-transfected M74-H cells showed a significant decrease in the number of colonies when compared to M74-mock cells (**Figure 6C**). Again, the colony numbers of the mutant M74-KLK7-S/A cells did not differ from those obtained with vector control cells (**Figure 6D**)**.** These findings indicate that the enzymatic activity of KLK7 reduces melanoma cancer cell proliferation and colony formation.

3.6 KLK7 overexpression alters cell morphology and modulates expression of cell adhesion molecules

Strikingly, both M74-D6 and M74-H cells overexpressing KLK7 displayed an elongated and irregular morphology with membrane protrusions typical of invading tumor cells not seen in M74-mock cells **(Figure 7A)**. Since KLK7 has been implicated in the shedding of adhesion molecules (Johnson et al., 2007; Ramani et al., 2008), we analyzed E-cadherin expression in KLK7-overexpressing cells *versus* vector control cells, applying an antibody that recognizes the extracellular region of E-cadherin. As shown in **Figure 7**, E-cadherin staining was markedly decreased in M74-D6 and M74-H cells, as indicated by a weak and diffuse staining, whereas a strong membrane-associated staining of E-cadherin was observed in the M74-mock cells (**Figure 7B, upper panel)**.

MCAM/CD146 (Melanoma Cell Adhesion Molecule) was originally identified as a marker of melanoma progression (Haass and Herlyn, 2005). Interestingly, MCAM/CD146 staining was significantly increased in KLK7-overexpressing M74-D6 and M74-H cells compared to M74-mock cells **(Figure 7B, lower panel)**. These results suggest that KLK7 induces downregulation of cell adhesion molecules (such as E-cadherin) thus triggering a more motile and invasive phenotype accompanied with a concomitant upregulation of other adhesion molecules (such as MCAM/CD146) involved in homotypic cell adhesion and interaction with the extracellular matrix.

3.7 KLK7 overexpression supports melanoma cell migration and invasion.

Because reduced E-cadherin expression may reflect the gain of migratory properties and promotion of cell invasion, we investigated the effect of KLK7 expression on cell motility using a modified Boyden chamber assay. While KLK7 overexpression repressed cell proliferation, it interestingly induced a significant increase in cell migration in both M74-D6 and M74-H cells compared to M74-mock cells **(Figure 8 A)**. M74-KLK7-S/A cells, expressing catalytically inactive KLK7, did not show any significant differences in cell migration compared to vector control cells **(Figure 8 B**).

Next, cellular invasion was assessed using an *in vitro* Matrigel invasion assay to determine whether the increased migration rate induced by KLK7 in melanoma cells was associated with increased invasive properties as well. For this, cells were seeded into the upper chamber of a Matrigel^{TM} coated filter, and serum-containing medium was used as chemoattractant in the lower chamber. Twenty-four hours after seeding, cells that invaded through the Matrigel substrate were quantified on the lower side of the filter. As shown in **Figure 8C**, KLK7-overexpressing M74-D6 and M74-H cells revealed a strong and significant increase in cell invasion relative to vector control cells.

4. Discussion

One of our main findings is that several KLKs are ectopically expressed in skin melanomas compared to normal melanocytes, which do not express any of the *KLK* genes analyzed. Particularly, KLK7 was found to be the major secreted kallikrein-related peptidase in melanoma *in vitro,* and was ectopically expressed *in vivo* in resected human melanoma tissues as well. In addition, melanoma cells that overexpress KLK7 exhibited a significant increase in cell migration and invasion. Thus, KLK7 may be an aberrantly expressed melanoma-produced proteinase with tumorigenic functions and may represent a potential key element in melanoma progression. Although KLK7 has been described as a biomarker in many types of cancers (Devetzi et al., 2013; Walker et al., 2014; Kryza et al., 2016), to our knowledge, this is the first report demonstrating a role for KLK7 in the metastatic process of melanoma. Our study showed that only 69 % of the analyzed melanoma cell lines ectopically express *KLK7*, whereas *KLK14* and *KLK6* are expressed by almost all of the cell lines. Nevertheless, when examined by ELISA, KLK7 was found to be the most abundantly secreted protein (up to 958 ng/L) into the culture media of the melanoma cells. The observed antigen levels were similar to those found in normal human epidermal keratinocytes (NHEK; ~1 µg/L) that constitutively express KLK7 (Hatano et al., 2013). *KLK7* expression was found both in primary melanoma cell lines (such as WM115, Dauv1, HM11) and in metastatic cell lines (such as SK-Mel 5 and MT10). In papillary thyroid cancer, KLK7 upregulation was found to be correlated to BRAF mutations (Kim et al., 2010). Although, BRAF, NRAS or C-Kit mutations have been identified as frequently mutated oncogenes in melanoma (Flaherty et al., 2012), no correlation was found between *KLK7* and the mutation status in our study (see Supplementary Table 2). This suggests that KLK7 upregulation in melanoma may be rather influenced by other factors from the tumor microenvironment. In line with this hypothesis, KLK7 has been shown to be upregulated when benign hyperplastic prostate epithelial cells were co-cultured with fibroblasts but not in separately cultured epithelial cells (Yang et al., 2010).

As demonstrated by immunohistochemistry, our analysis clearly show that KLK7 is highly expressed in both primary melanoma and in metastatic melanoma tissues, but only low KLK7 levels, if at all, are seen in nevi or in the normal melanocytes in the healthy skin, from which these cancers may arised. This is in line with the failure to detect any KLK7 mRNA in primary normal human epidermal melanocytes (NHEM) by PCR. The melanoma tumor specimens were only investigated by immunohistochemical techniques and not by RT-PCR because the melanoma specimens contain keratinocytes of the stratum corneum and stratum granulosum which are a major source of KLK7

(Yousef et al., 2000; Egelrud et al., 2005). Interestingly, we observed a significant trend between KLK7 staining intensity and the severity of the disease. The percentage of cells displaying strong staining intensity (13.5 %) or moderate (25 %) was higher in metastatic melanomas compared to primary melanomas (only 3.5 % and 18 %, respectively). Therefore, KLK7 might be considered as a potential progression marker for skin melanoma. In concordance with our findings, a previous gene expression study revealed that *KLK7, KLK4* and *KLK11* expression is correlated to metastastic dissemination and associated with overall survival of patients with primary cutaneous melanoma (Winnepenninckx et al., 2006). In our study only one benign nevus (out of six) weakly stained for KLK7. Whether the weak KLK7 staining could display clinical significance (*i.e.* detection of early melanocytic transformation) is not yet clear but deserves to be further explored. Interestingly, KLK7 has been found to be a useful biomarker to distinguish various types of kidney tumors when morphology is similar (Gabril et al., 2010).

In line with our detection pattern, more recently, KLK7 expression has been found to be increased in atypical nevi and primary melanoma compared to its expression in common nevi (Martins et al., 2011; Rezze et al., 2011). However, the study by Martins and co-workers did not show significant differences between KLK7 staining in primary and metastatic tissues (Martins et al., 2011). The differences concerning KLK7 staining intensities in metastatic tissues *versus* primary melanoma in our study and in the study of Rezze *et al.* may be due to the sensitivity/specificity of the techniques used for detection, statistical analysis methods, and/or the specimen selection (cohorts). Of note, Rakosy *et al.* found that KLK7 was down-regulated in ulcerated primary melanoma compared with non ulcerated tissues (Rakosy et al., 2013). The occurrence of KLK7 mRNA variants could also explain the existence of different expression patterns. Indeed, it has been reported that some KLKs including KLK7 use alternative promoters to generate organ- and disease-specific transcripts (Dong et al., 2008).

Some KLKs have been shown to signal through multiple pathways to stimulate cell proliferation. (Gratio et al., 2010; Gratio et al., 2011; Chung et al., 2012; Filippou et al., 2015; Kryza et al., 2016). KLK7 involvement in cell growth has also been demonstrated in different cancers. Indeed, simultaneous expression of KLK4, KLK5, KLK6, and KLK7 in ovarian cancer cells resulted in an increase of tumor burden *in vivo* (Prezas et al., 2006), KLK7 increased esophageal adenocarcinoma cell proliferation (Xi et al., 2015), and we have recently revealed that in colon cancer cells overexpressing KLK7 proliferation was increased *in vitro* and, moreover, these cells developed larger tumors in nude mice (Walker et al., 2014). Surprisingly, in the present study we found that KLK7 overexpression strongly suppressed cell proliferation and colony formation of melanoma cells *in vitro.* The observed inhibitory effect on cell proliferation suggests that KLK7 may exert opposite effects depending on the cancer cell-type. The KLK7-mediated reduction of proliferation may suggest a common mechanism shared with some other KLKs under certain conditions, which also trigger antiproliferative effects in prostate cancer (Veveris-Lowe et al., 2005), ovarian cancer (Prezas et al., 2006; Pepin et al., 2011), and breast cancer cells (Sotiropoulou et al., 2009).

Since previous studies have shown that KLK7 mediates both proteolytic and non-proteolytic functions in ovarian cancer (Dong et al., 2014; Kryza et al., 2016), we used an enzymatically inactive KLK7 mutant, where the active-site serine residue has been replaced by alanine. The mutant KLK7-S/A did not affect cell proliferation and did not induce cell migration (Figure 6B, D and Figure 8B), suggesting that KLK7 effects are mostly mediated by its proteolytic activity. In cell biological experiments, we were unable to directly follow the enzymatic activity of secreted KLK7 into the cell media, most likely because of the low enzyme concentration (≈38 pM, Figure 2). Thus, the produced KLK7 levels seem sufficient to mediate cell responses (cell migration and cell invasion) but not to detect KLK7 enzymatic activity using fluorogenic chymotrypsin substrates which requires much higher enzyme concentrations in the nanomolar range (data not shown). To enable studies of the enzymatic activity of proteases involved in cell signaling, new highly sensitive tools have to be developed. Nevertheless, the KLK7 axis, which depends on enzymatic activity, represents a promising target and calls for further *in vivo* studies and also for the development of selective inhibitors of KLK7.

The mechanism by which KLK7 inhibits cell proliferation is not clear but may be due to cell cycle arrest or processing of repressive factors (cytokines) such as IL-1beta, a known substrate of KLK7 (Nylander-Lundqvist and Egelrud, 1997) that has been shown to inhibit melanoma growth (Neville et al., 1990). Accumulating evidence indicates that EMT, often accompanied by loss of E-cadherin expression, plays a critical role in metastasis, whereby the genes involved in EMT promote cell migration and invasion but suppress proliferation of melanoma (Haass and Herlyn, 2005; Pearlman et al., 2017). In pancreatic cells, KLK7 induced shedding of E-cadherin (Johnson et al., 2007) and induced EMT in prostate cancer (Mo et al., 2010) associated with a remarkably increased cell migration and invasion. In our study, we found that KLK7 overexpression induced changes in cell morphology and a significant decrease in E-cadherin associated with an increase of MCAM/CD146 expression. Melanoma cells do not show a classical epithelial or mesenchymal phenotype (Kim *et al*., 2013). In fact, analysis of EMT markers did not reveal significant differences in vimentin and N-cadherin expression in M74-control cells and KLK7-overexpressing cells (Figure S4). These data suggest that the observed KLK7-mediated decrease of E-cadherin is not due to an EMT process but may be due to cleavage/degradation of the extracellular domain by the secreted KLK7. The KLK7-mediated loss of E-cadherin and gain in MCAM/CD146 may suggest a possible role of KLK7 in melanoma cell aggregation, a known mechanism of chemoresistance in cancer.

Although cell proliferation is the hallmark of most cancers, invasion and proliferation are uncoupled in melanoma, such that highly proliferative melanoma cells are less likely to be invasive, and *vice versa*, a phenomenon called "phenotype switching" (Hoek et al., 2008). It has been suggested that tissue microenvironmental factors signal the switch of some cells to an invasive phenotype. Thus, in addition to genetic heterogeneity, acquired phenotype heterogeneity contributes to therapy failure with the currently used MAP-kinase pathway inhibitors. To identify the role of KLK7 on melanoma progression, we measured the *in vitro* invasive potential. Our results showed that KLK7 overexpression led to a more migratory and more invasive phenotype. This observed KLK7-induced effect on the dissemination process is consistent with the described role of KLK7 in cell invasion (Prezas et al., 2006; Johnson et al., 2007; Mo et al., 2010) and its association to unfavorable prognosis in several types of cancers (Dong et al., 2010; Kryza et al., 2016).

The mechanisms underlying the KLK7-mediated increase of cell motility and invasion remain unknown but might be directed by modulating other enzymatic pathways important in cancer metastasis. Previous studies showed that KLK7 also cleaved pro-MMP9 generating active MMP9 (Ramani et al., 2011), and urokinase type plasminogen activator receptor (uPAR) (Ramani and Haun, 2008), two members of key proteolytic systems in melanoma invasion (Frohlich, 2010). However, it is unlikely that MMP9 is involved in the KLK7 effect seen in our studies, since analysis of protease array data as well as Western blot analysis using conditioned media from control M74-cells and from KLK7-transfected M74-cells failed to detect any MMP9 protein (data not shown). Although the pathways responsible for KLK7-mediated stimulation of melanoma cell migration and invasion are beyond the scope of this report, it is worth to note that using protease array techniques, an up-regulation of members of the cathepsin family, certain matrix metalloproteinases, and proteases inhibitors was observed in cell culture supernatants of melanoma cells that overexpress KLK7 (data not shown). Because proteases often act indirectly by inducing other proteases or other factors, more global approaches such as arrays and/or proteomic, peptide mapping and degradomic analysis may have to be applied in order to identify KLK7 substrates or specific pathways which are activated by KLK7.

In conclusion, we have shown that KLK7 is an aberrantly expressed melanoma-produced proteinase and that KLK7 decreases cell proliferation and colony formation but induces an increase in cell motility and cell invasion. Our results, together with those from previous reports, indicate a correlation of KLK7 expression with melanoma metastasis and suggest that KLK7 is a potential biomarker for melanoma progression.

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Conflicts of Interest

None

Author's contributions

Conception and design: D. D.

Technical support and access to equipment: T. D., M. H., S. A., F. S. and D.D.

Acquisition of data: D. D.

Material support: M. B., C. E.A, F. S and V.M., E. P. D,

Analysis and interpretation of data: L.D.′ F.W., V.M. and D. D.

Writing the manuscript: V.M. and D. D.

Review, and revision of the manuscript: D. D., V.M., L.D., F.W. and M. B.

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Table 1: KLK mRNA expression levels in human melanoma cell lines analyzed by semi-quantitative RT-PCR.

Cell line	**Gene**						
	KLK4	***KLK5***	***KLK6***	***KLK7***	***KLK8***	***KLK10***	***KLK14***
Colo 792							
MEWO							
501 Mel							
A375							
Colo 829							
Colo 794							
Dauv1							
M74							
MM170							
SK-Mel5							
SK-Mel28							
WM115							
WM266-4							
C8161							
HM11							
Sbcl2							
WM1361							
WM852							
XP44RO							
MM 127							
MT10							
M230							
WM1791c							
Positive expression (% of 23 cell lines)	**30**	**34**	**86**	**60**	**13**	**39**	**100**
NHEM*							
HaCaT**							

Absent or very weak	Moderate	High

*, normal human embryonic melanocytes (NHEM), used as a negative control;

** human keratinocyte-like HaCaT cells, used as a positive control

Figure legends

Figure 1: Analysis of the mRNA expression pattern of KLK5, 6, 7, and 14 in different melanoma cell lines by RT-PCR

Analysis was performed using RNA isolated from the indicated human melanoma cell lines. mRNA expression of KLK5, 6, 7, and 14 was characterized by semi-quantitative PCR as described in the Material and Methods section.*, Normal human embryonic melanocytes (NHEM), used as negative control; ** human keratinocyte-like HaCaT cells, used as positive control and GADPH as internal control.

Figure 2: Quantification of the antigen levels of various KLKs in the cell culture supernatants of human melanoma cell lines by ELISA.

(A) Secretion of KLK 4, 5, 6, 7, 8, 10, 13 and 14 in conditioned medium from 23 melanoma cell lines was assessed by ELISA and is presented in a scatter plot. Protein values represent the mean concentration of KLKs (µg/L) secreted by 10^6 cells, which were cultured for 24 h.

(B) Conditioned medium was collected from 23 melanoma cell lines and KLK7 concentration assessed by ELISA. Protein values represent the mean concentration of KLK7 (µg/L) secreted by 10^6 cells, which were cultured for 24 h. Note that normal melanocytes (NHEM cells) express very low, if any, KLK7.

Figure 3: Detection of KLK7 on formalin-fixed, paraffin-embedded sections of primary and metastatic melanomas by immunohistochemistry.

(A) Representative images of the KLK7 expression pattern in human melanocytic lesions obtained by immunohistochemical staining. **(a)** Nevi with negative staining (intensity: 0); **(b, c)** primary melanoma with weak KLK7 staining (intensity: +1); **(d, f)** metastatic melanoma with moderate to strong KLK7 staining (intensity: +2 and +3); (**e)** primary melanoma with strong KLK7 staining (intensity: +3). Note that there is no staining in the nevi, but a significantly increased staining in primary (PM) and metastatic melanoma (MM). Immunohistochemical staining generally exhibits a heterogeneous cytoplasmic KLK7 expression regardless of the stage (compare **b, c and e** *versus* **d and f**). As expected, normal-appearing epidermal cell layers adjacent to nevi (**a)** or to primary melanoma **(b)** show the highest KLK7 levels in the stratum corneum layers (white stars) (Yousef et al., 2000). Black arrows in (b), (d), (e), and (f) point to positive KLK7 immunoreactivity in the cytoplasmic compartment of the cells. The grey arrow in (a) points to a normal melanocyte with negative staining. Black stars in (c) indicates KLK7 staining in the vessels. Scale bar = 200 µm.

(B) Staining intensity of KLK 7 was categorized into intensity ranges from 0 to 3+. The average percentage of cells in each group was determined and is shown in a stacked graph for each tissue type (n = 38). N, nevi; PM, primary melanoma; MM, metastatic melanoma

Figure 4: KLK7 triggers ERK1/2 phosphorylation in melanoma cells.

(A) Immunoblot detection of phosphorylated ERK1/2 (upper panel) in quiescent MeWo cell lysates treated with or without KLK7 (10 nM) for the indicated time periods. To confirm equal protein loading, the membranes were stripped and incubated with an antibody detecting both non-phosphorylated and phosphorylated ERK1/2 (lower panel). Results are representative of two separate experiments.

(B) Dose-dependent activation of ERK1/2 phosphorylation by KLK7. Quiescent MeWo cells were stimulated with the indicated concentrations of KLK7 for 10 min. The upper panel shows the result obtained with an antibody specifically directed to phosphorylated ERK1/2 and the lower panel the result using an antibody detecting both non-phosphorylated and phosphorylated ERK1/2. Results are representative of two separate experiments.

Figure 5: Quantification of KLK7 mRNA and measurement of KLK7 protein secretion in melanoma M74-derived cell lines.

Melanoma M74 cells were stably transfected with the KLK7 expression plasmid pRcRSV-KLK7 or with the pRcRSV vector alone. M74-D6 represents a selected clone overexpressing KLK7, M74-H is a batch-transfected M74-derived KLK7-overexpressing cell line. M74-mock, M74 cells, stably transfected with the empty vector only.

(A) Total RNA from either M74-D6, M74-H, or M74-mock cells was reverse transcribed and subsequently analyzed by SYBR Green fluorescence-based QPCR to quantify the KLK7 mRNA expression levels. *GAPDH* was used as the endogenous reference gene for normalization of KLK7 expression.

(B) Conditioned medium was collected from either M74-D6, M74-H, or M74-mock cells after 24 h and KLK7 antigen levels were assessed by ELISA. The inset depicts the KLK7 antigen levels measured in cell culture supernatants of HaCaT cells. Data represent the mean protein values ± SEM of three independent experiments.

Figure 6: Overexpression of wild-type KLK7, but not the active-site mutant KLK7-S/A, inhibits cell growth and colony formation in melanoma cells.

(A) M74-D6, M74-H, and M74-mock cells were seeded as described in Materials &Methods and counted after 96 h. Data are given as mean ± SEM of three independent experiments. $**P < 0.01$, M74-D6 and M74-H cells *vs*. M74-mock cells.

(B) M74-cells overexpressing wild-type KLK7 (M74-H), or its active-site mutant (M74-KLK7-S/A), or M74-vector cells (M74-mock) were seeded as described in Materials &Methods and proliferation was analyzed after 72 h using the WST-1 Cell Proliferation Assay Kit. Data are given as mean ± SEM of three independent experiments. M74-H *vs*. M74-Mock,$***P < 0.001$; M74-KLK7-S/A *vs*. M74-Mock, NS ($P>0.05$).

(C) M74-D6, M74-H, and M74-mock cells were plated as described in Materials &Methods and incubated for two weeks. KLK7 induced a strong decrease in melanoma colony formation. Representative images (upper panel) were captured and quantified (lower panel) with an Image Quant™ LAS 4000 digital imaging system. Columns represent the mean percentage of colonies ± SEM of three independent experiments, each performed in duplicate. $***P<0.001$.

(D) M74-cells overexpressing wild-type KLK7 (M74-H), or its active-site mutant (M74-KLK7-S/A), or M74-vector cells (M74-mock) were plated as in (C). Columns represent the mean percentage of colonies ± SEM of three independent experiments, each performed in duplicate. M74-H *vs*. M74-mock, $***P < 0.001$; M74-KLK7-S/A *vs*. M74-mock, NS ($P>0.05$).

Figure 7: KLK7 overexpression in M74 cells results in altered cell morphology and modulates expression of cell adhesion molecules.

(A) Representative phase-contrast images of KLK7-overexpressing cells: both the clonal M74-D6 and the batch-transfected M74-H KLK7 overexpressing cell lines reveal an altered cell morphology compared with M74-mock vector control cells. Cells overexpressing KLK7 display a spindle-shaped morphology with membrane protrusions. Scale bar = 50 µm.

(B) Representative immunofluorescence images (green) show a strong decrease of E-cadherin (upper panel) and increase of MCAM/CD146 (lower panel) staining in KLK7-overexpressing M74-D6 and M74-H cells compared to M74-mock cells. Nuclear staining was performed with DAPI (blue). Scale bar = 100 µm.

Figure 8 : KLK7 overexpression but not KLK7 mutant induces melanoma cell migration and invasion.

(A) M74-D6, M74-H, and M74-mock cells were plated on 8-µm pore-size Transwell® inserts. After 24 h, cells were stained with crystal violet/methanol and migrating cells were counted using an optical imaging system. Migrated cells from six filter fields were calculated with the Image-J software. **, *P* <0.01; ***, *P* <0.001.

(B) M74-cells overexpressing wild-type KLK7 (M74-H), its active-site mutant (M74-KLK7-S/A), or M74-vector cells (M74-mock) were plated and analyzed as described in (A). M74-H *vs*. M74-mock, ****P* < 0.001; M74-KLK7-S/A *vs*. M74-mock. NS (P>0.05).

(C) Transwell invasion assay: M74-D6, M74-H, and M74-mock cells were plated on 8-µm pore-size Transwell® inserts precoated with 10 µg of Matrigel™ (5×10^4 per well) and invasion of the cells analyzed as described in (A). ***, P <0.001.

Supplementary Figure Legends

Figure S1: Sequence of KLK7-S/A in pRcRSV

Nucleotide and amino acid sequence (given as one letter code) of the KLK7 mutant carrying the serine to alanine exchange (indicated in red) within the catalytic triad.

Figure S2 : QPCR analysis of KLK7 mRNA expression in a subset of melanoma cell lines and in normal melanocytes

Real-time PCR analysis of KLK7 expression in the indicated melanoma cell lines and in melanocytes NHEM#1 (PromoCell) and NHEM#2 (Cascade Biologics). Data were normalized by the amount of GAPDH mRNA and are means ± SD from duplicate data.

Figure S3: KLK7 triggers p42/p44 MAP kinase (ERK1/2) phosphorylation in MeWo melanoma cells but not Smad2 or Stat3

(A) MeWo cells (A wild type model for studying signal pathways) were grown to 70% confluence and then serum-starved for 48 h. Quiescent cells were treated with active recombinant KLK7 (10 and 20 nM) for 10 min. Cells were lysed and analyzed by Western blot for phospho-Smad2 (ser 465/467) (upper panel), phospho-Stat3 (tyr705) (middle panel) and phospho-Erk1/2 (lower panel). To confirm equal protein loading, the membranes were stripped and incubated with antibodies detecting both non-phosphorylated and phosphorylated Smad2/3 (upper panel), Stat3 (middle panel) and ERK1/2 (lower panel).

Note that KLK7 phosphorylates only ERK1/2 in MeWo cells and not Smad2 (which activation is mediated by TGF-β pathway) or Stat3 (which activation is mediated by cytokines and growth factors).

B) M74-Mock cells and KLK7 overexpressing cells (M74-D6 and M74-H) were grown to 80% confluence and then serum-starved for 48 h. Cells were lysed and analyzed for phospho-Smad2 (ser 465/467) (upper panel), phospho-Stat3 (tyr705) (middle panel) and phospho-Erk1/2 (lower panel). To confirm equal protein loading, the membranes were stripped and incubated with antibodies detecting both non-phosphorylated and phosphorylated Smad2/3(upper panel), Stat3 (middle panel) and ERK1/2 (lower panel).

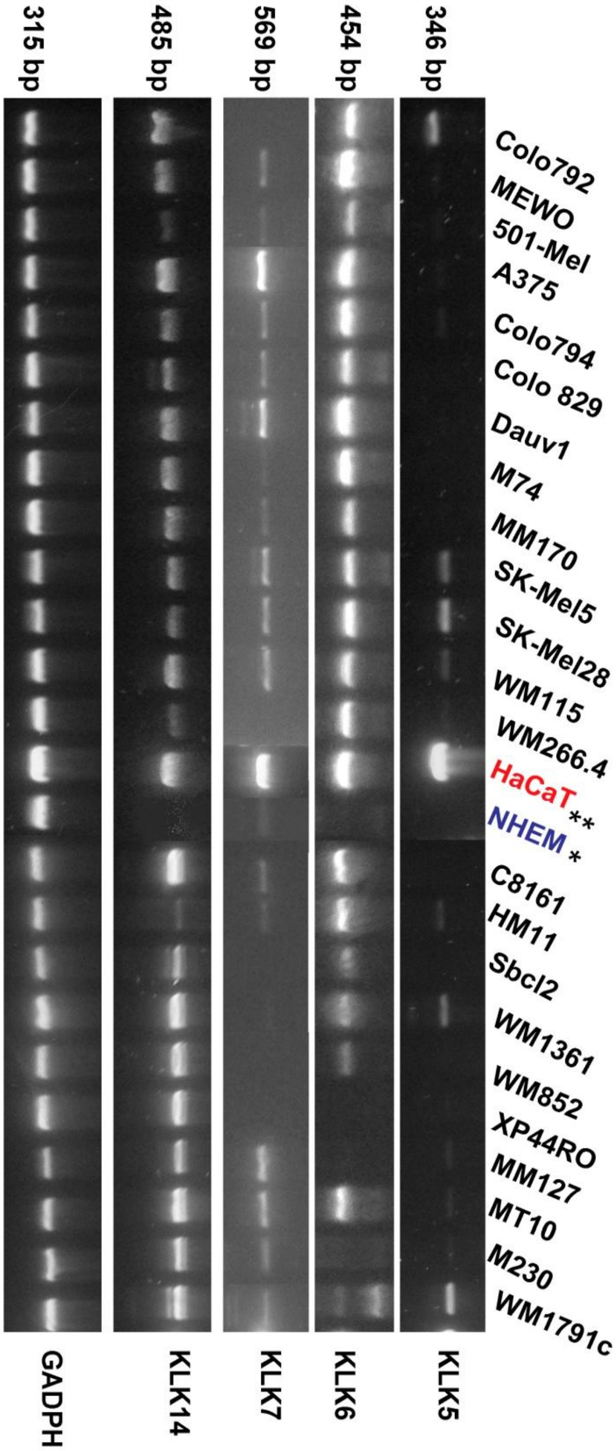
Note that as expected ERK1/2 is constitutively phosphorylated in all cells, which is the consequence of the BRAF mutation in M74 cells. Neither Smad2 phosphorylation nor Stat3 phosphorylation were detected under our conditions. For validation of the phospho-Smad2 and phospho-Stat3 antibodies, FCS was used as positive control for kinase-phosphorylation.

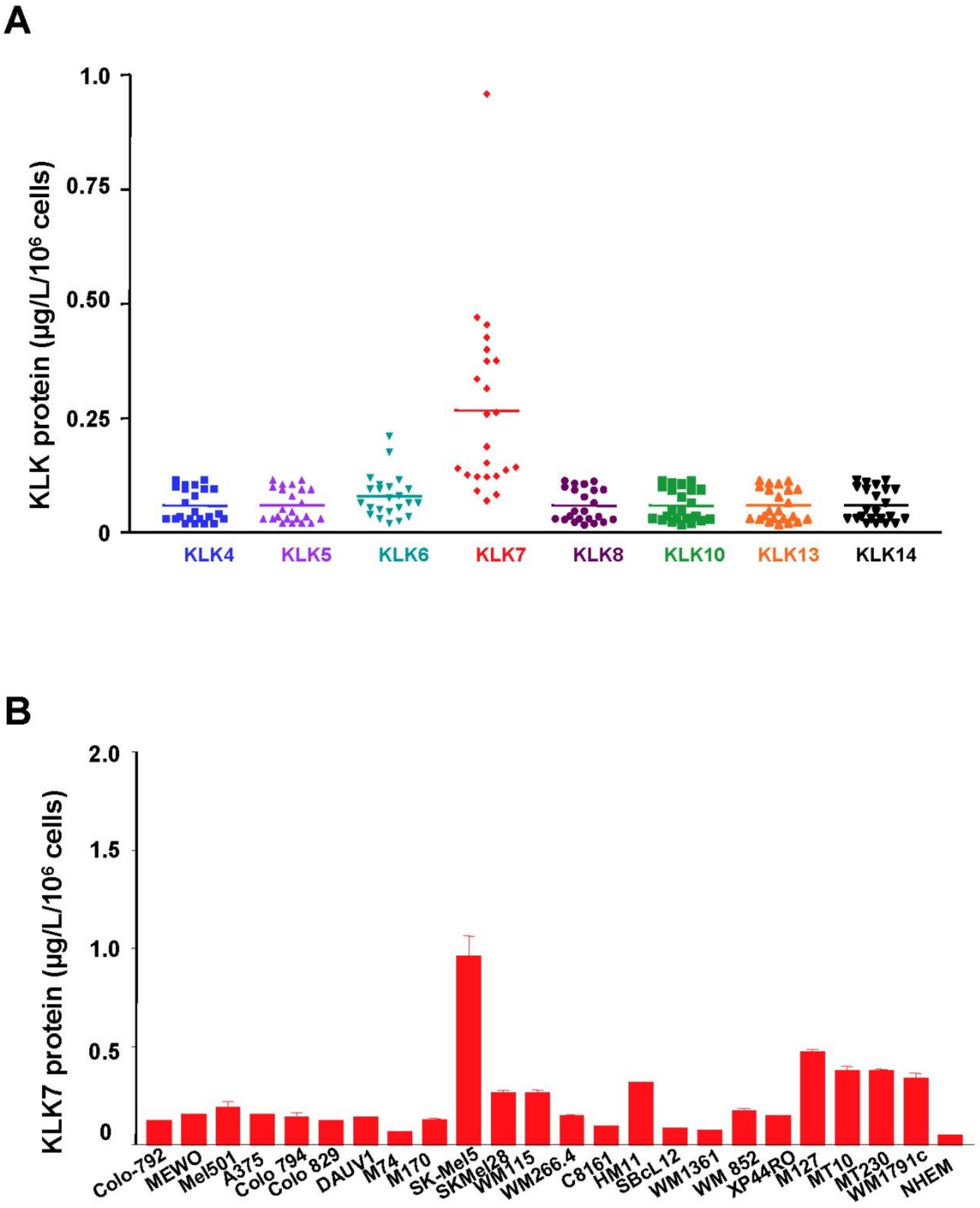
Figure S4 : Analysis of EMT expression markers in KLK7-overexpressing cells

Whole cell extracts from M74-mock or KLK7-expressing cells (M74-D6 and M74-H) were tested by Western Blot for E-cadherin, N-cadherin, vimentin and snail expression using rabbit monoclonal anti-N-cadherin (D4R1H) (#13116), mouse anti-E-cadherin (32A8) (#5296), rabbit monoclonal anti-snail (C15D3) (#3878) (Cell Signaling Technologies, Beverly, MA) and mouse anti-vimentin (Clone V9) (Santa Cruz Biotechnology, Santa Cruz, CA). To confirm equal protein loading, the membranes were stripped and incubated with monoclonal anti-β-actin [AC-15] (#Ab 6276) (Abcam, Cambridge, UK) (lower panel). Note that a slight reduction of E-cadherin, whereas no changes of vimentin or N-cadherin levels are observed. Snail expression is not detected in M74-mock cells and in KLK7-

expressing cells (M74-D6 and M74-H). HCT116 cells were used as positive control for snail expression. Results are representative of two separate experiments.

Figure 1

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0.25
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KLK5
KLK6
KLK7
KLK8
KLK10
KLK13
KLK14
B
2.0
1.5
1.0
0.5
0
KLK7 protein (µg/L/10⁶ cells)
Colo-792
MEWO
Mel501
A375
Colo 794
Colo 829
DAUV1
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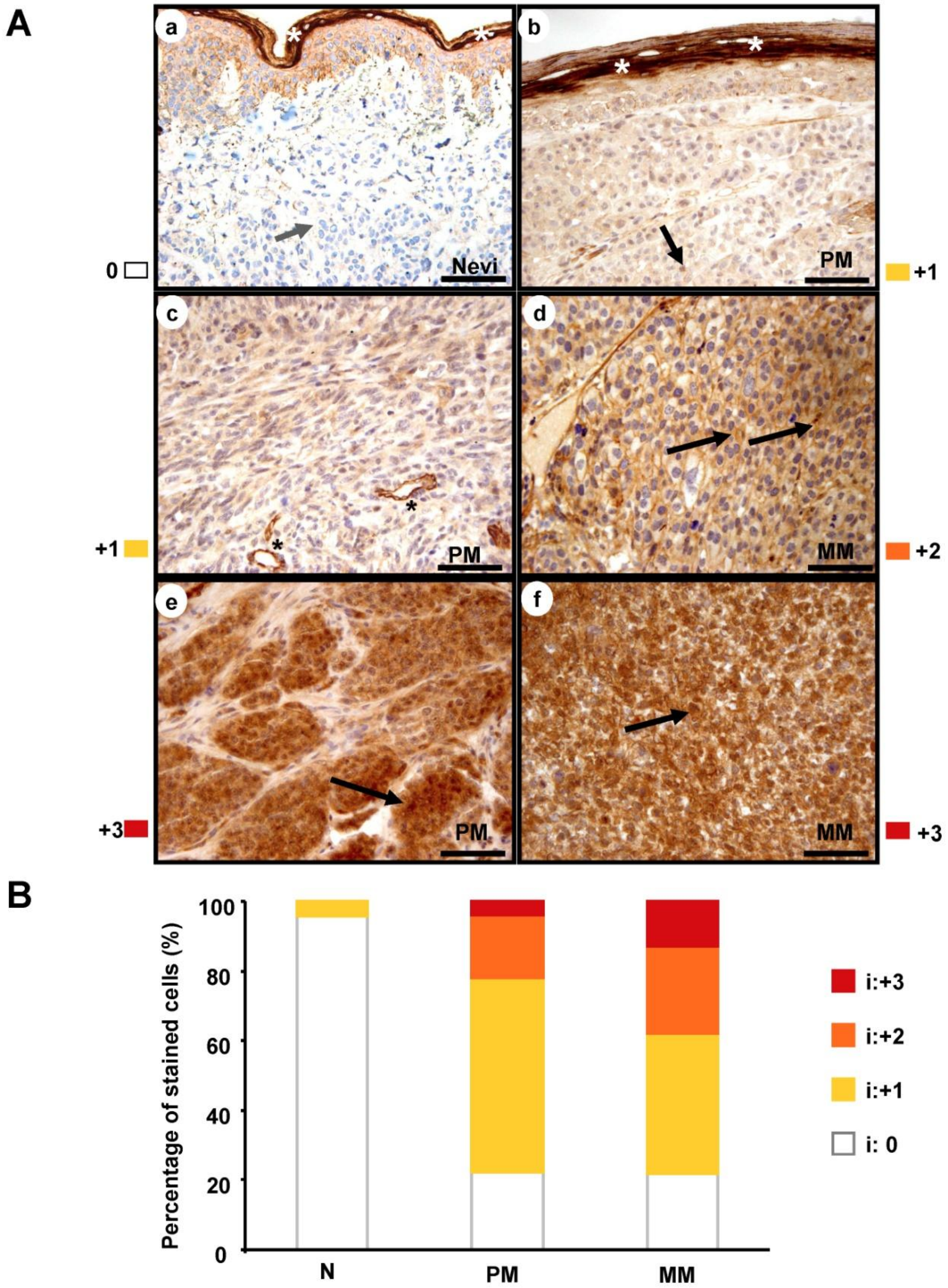
Figure 2


A
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*
*
Nevi
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b
*
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PM
🟨 +1
c
*
*
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MM
🟧 +2
e
PM
+3 🟥
f
MM
🟥 +3
B
Percentage of stained cells (%)
100
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60
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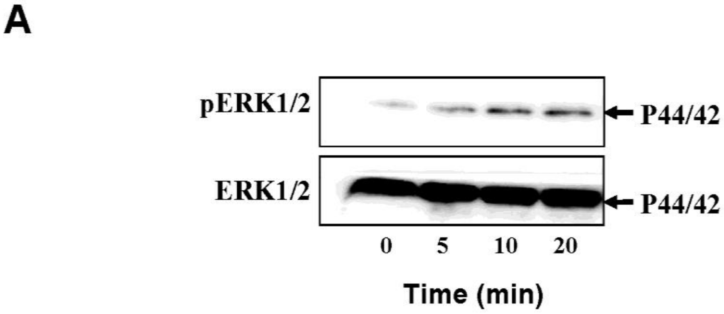
Figure 3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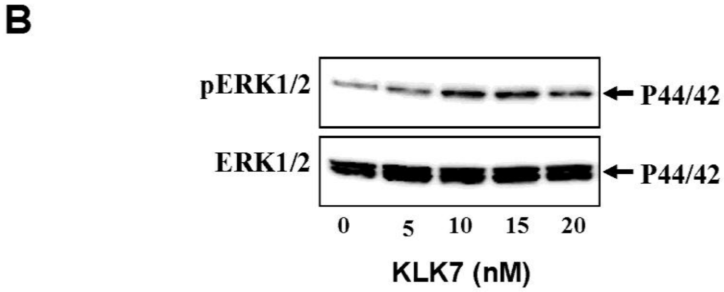
5
10
20
Time (min)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5
10
15
20
KLK7 (nM)

Figure 4

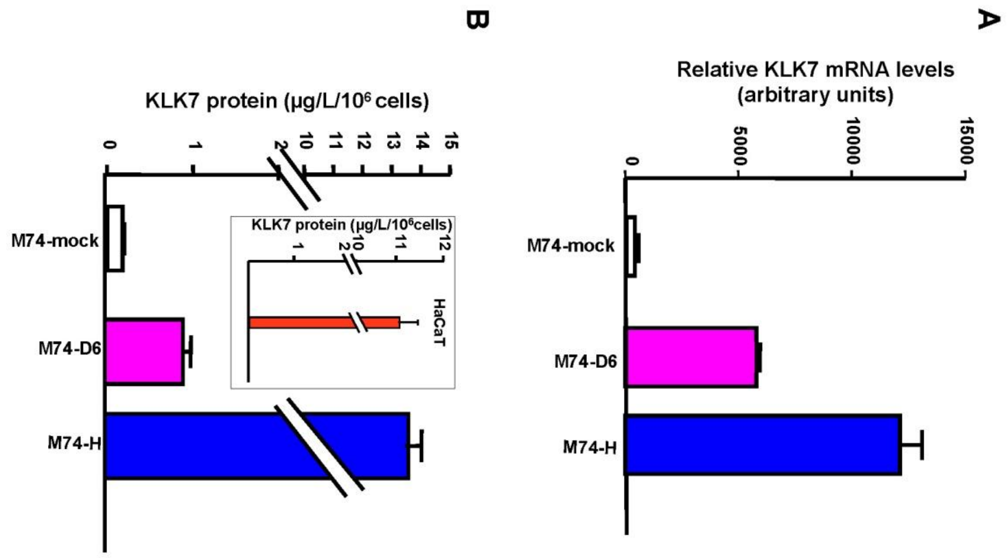


Figure 5

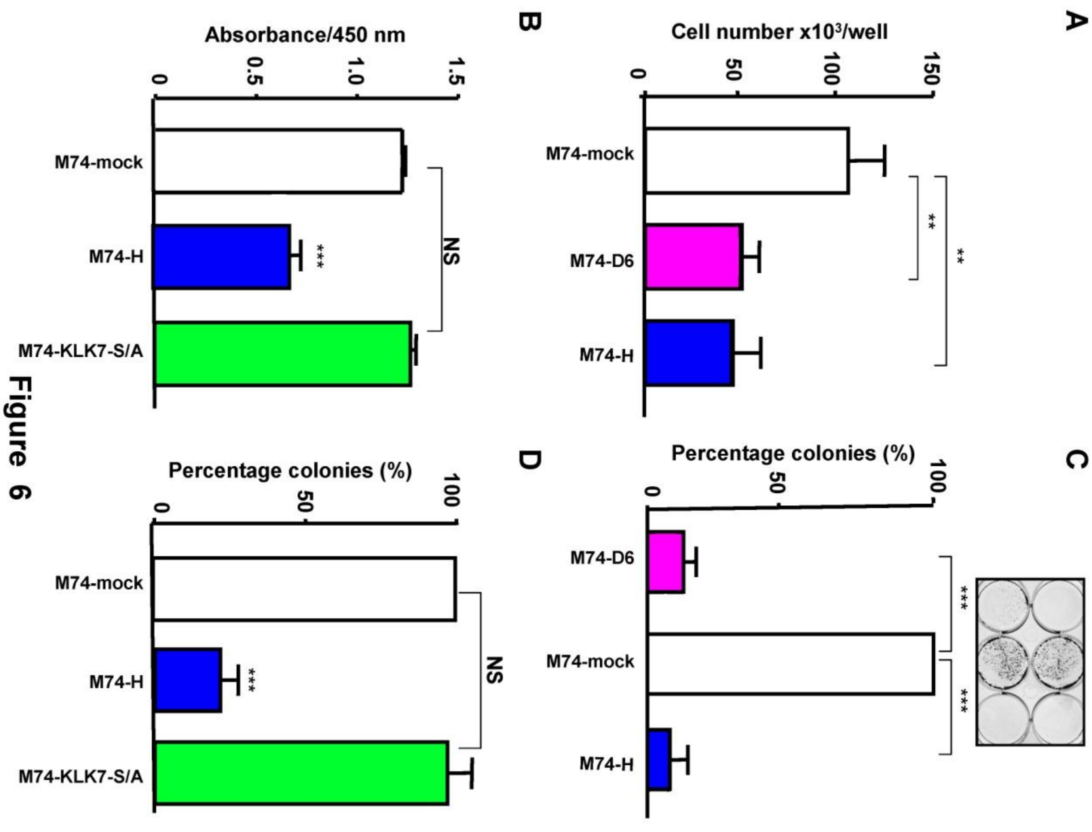


Figure 6

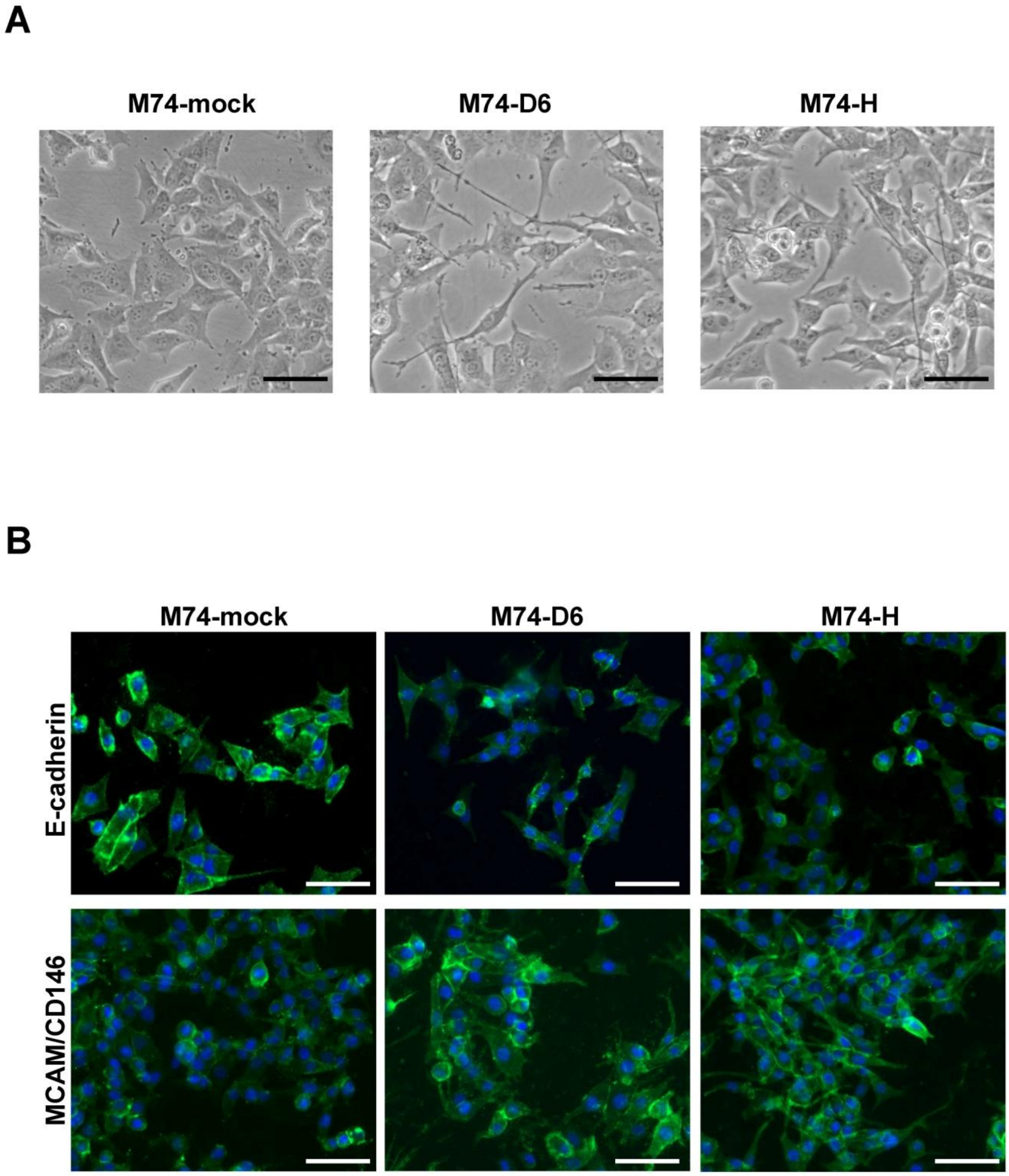
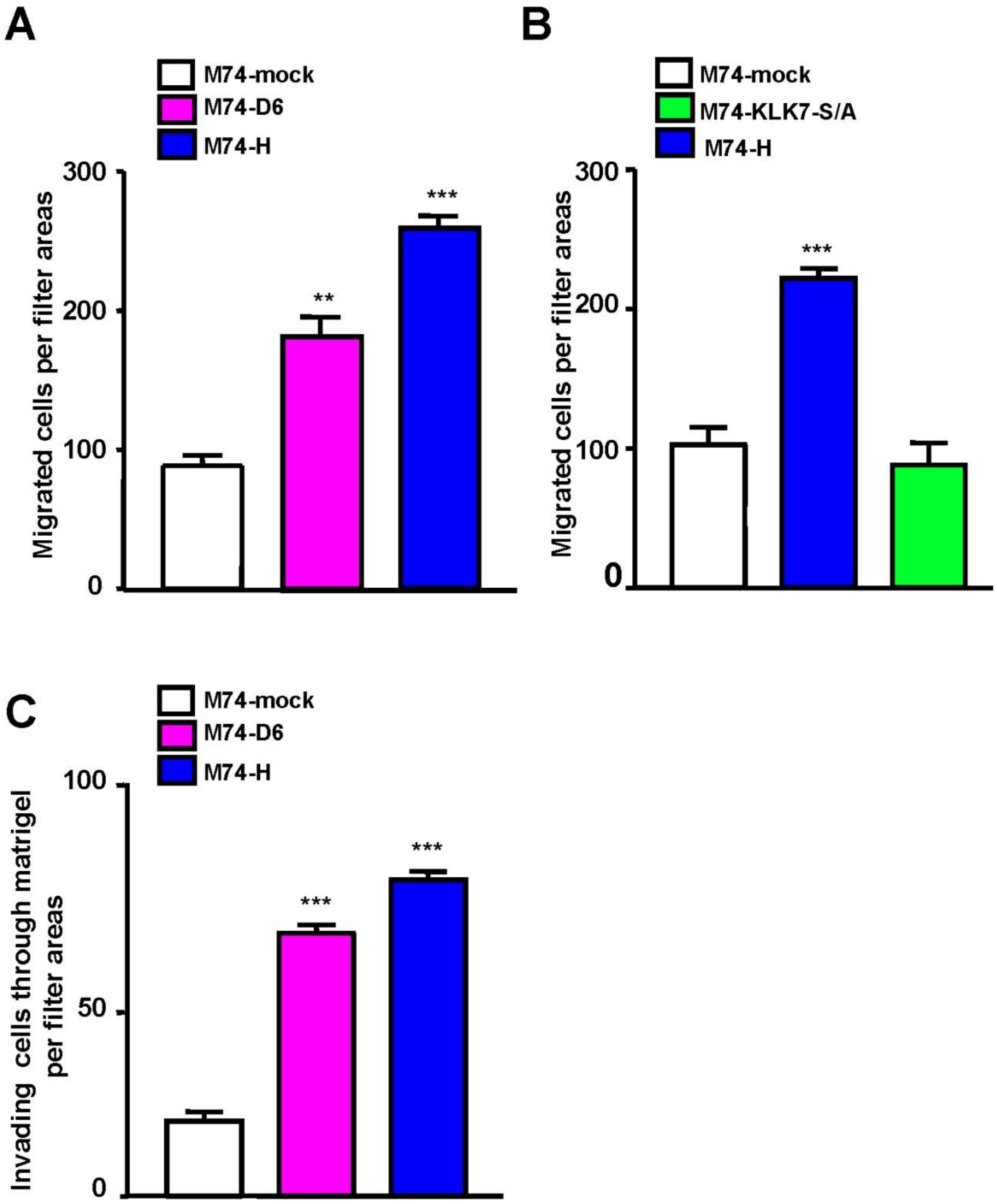

A
M74-mock
M74-D6
M74-H
B
M74-mock
M74-D6
M74-H
E-cadherin
MCAM/CD146

Figure 7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M74-mock
M74-D6
M74-H
Migrated cells per filter areas
300
200
100
0
**
***
B
M74-mock
M74-KLK7-S/A
M74-H
Migrated cells per filter areas
300
200
100
0
***
C
M74-mock
M74-D6
M74-H
Invading cells through matrigel per filter areas
100
50
0
***
***

Figure 8