Klinische Forschergruppe der Frauenklinik und Poliklinik der TU München

Klinikum rechts der Isar

OV-MZ-6 ovarian cancer cells overexpressing kallikrein-related peptidases KLK4, 5, 6 and 7: effects on expression of cancer-related genes

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Vollständiger Abdruck der von der Fakultät für Medizin

der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Medizin (Dr. med.)

genehmigten Dissertation.

Vorsitzender: Prof. Dr. Ernst J. Rummeny

Prüfer der Dissertation:

1. Prof. Dr. Viktor Magdolen
2. Prof. Dr. Marion B. Kiechle

Die Dissertation wurde am 24.03.2016 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 03.08.2016 angenommen.

To My Father

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1 Introduction

1.1 Ovarian cancer

Ovarian cancer accounts for approximately 3% of all cancers in women and is the fifth leading cause of cancer-related death among women in the United States [1]. Based on the origin of the cancer cells, ovarian cancer can be classified into epithelial tumors, germ cell tumors, sex cord stromal tumors, low malignant potential tumors, and mixed tumors. Epithelial carcinoma is the most common type, which includes serous tumors, endometrioid tumors, mucinous cystadenocarcinomas, Brenner tumors, and transitional cell tumors [2]. Ovarian cancer is staged according to the FIGO system using the information obtained from surgery [2-4, **Appendix 10.1**]. The most frequent stage at diagnosis is stage III, detected in over 70% of diagnoses [3].

At the early stages of ovarian cancer (FIGO stage I/II), symptoms are usually not apparent. These symptoms include bloating, pelvic pain, abdominal swelling, and/or urinary symptoms [5]. Diagnosis of ovarian cancer is based on physical examination (including pelvic examination), blood test (CA-125), and transvaginal ultrasound (TVUS) [3]. A rectovaginal examination may support surgery planning [6]. The final diagnosis must be confirmed histopathologically. During surgery staging is performed by inspecting the abdominal cavity, taking biopsies and looking for cancer cells in the abdominal fluid [3]. The treatment of ovarian cancer includes surgery, chemotherapy, and sometimes radiotherapy. The goal of the primary radical surgery is to remove all visible tumors and therefore the extent of the operation depends on the cancer stage. Ovarian cancer surgery includes at least removal of one (unilateral oophorectomy) or both ovaries (bilateral oophorectomy), of the Fallopian tubes (salpingectomy), of the uterus (hysterectomy), and of the omentum (omentectomy) as well as lymphadenectomy [3,6].

As shown in **Table 1**, the survival rates for early-stage ovarian cancer are high but are extremely low for late-stage ovarian cancer [7]. Therefore, detection of ovarian cancer at early stages is of critical importance for the patients. Screening for any types of cancers must be accurate and reliable, excluding false positive results [8].

Table 1. Five-year survival rates of patients with different stages of ovarian cancer

FIGO stage	5-year survival rate
I	90-95%
II	70-80%
III	20-50%
IV	1-5%

Data are taken from [7].

CA-125 was first isolated by Robert Bast in 1981 [9]. Most patients (90%) with advanced ovarian cancer have elevated serum CA-125 concentrations, but only 50% of early-stage patients have increased CA-125 levels [10]. Besides CA-125 is not specific for ovarian cancer, as CA-125 serum increases are also observed in patients with cancers of the breast, endometrium, gastrointestinal tract, and lung [11]. The low incidence rates of ovarian cancer and the lack of sensitivity and specificity limits the clinical use of CA-125 detection for ovarian cancer. However, monitoring the serum CA-125 levels is useful for predicting the treatment effects and patients' prognosis of ovarian cancer. CA-125 levels will decrease when the treatment is effective and the persistence of high levels of CA-125 during therapy is associated with poor survival rates in patients [12-16].

TVUS uses the ultrasound transducer in the vagina to visualize the organs within the pelvic cavity. TVUS can detect if there is a tumor in the ovary, but it cannot give the definite diagnostic information [17]. HE4, also called WAP four-disulfide core domain protein 2, is a protein encoded by the WFDC2 gene. It is also a tumor marker for ovarian cancer [18,19]. Due to the limitations of the above mentioned screening tests, it is of critical clinical importance to find more effective markers and treatment options for ovarian cancer. Cancer cells are also affected by the tumor microenvironment, including extracellular matrix and stromal cells. Proteases can regulate the tumor microenvironment, on one hand, by degrading the extracellular matrix and, on the other, by regulating signaling pathways by cleavage of substrates, *e.g.* cytokines, growth factors, and receptors via limited proteolysis. Recent studies indicate that some proteases, including kallikrein-related peptidases, show high expression in ovarian cancer tissue and correlate with patients' prognosis. Thus, these proteases could be potential markers for ovarian cancer.

1.2 Tissue kallikrein-related peptidases

The two true kallikreins, plasma and tissue kallikrein, respectively, are serine proteases and liberate kinins (bradykinin and kallidin) from kininogen, peptides responsible for the regulation of blood pressure and activation of inflammation [20]. The KLKB1 gene encoding plasma kallikrein is located on chromosome 4q34-35. Plasma kallikrein may not only target kininogen, since it is also capable of generating plasmin from plasminogen [21,22]. Tissue kallikrein (KLK1) is member of the kallikrein-related peptidase family consisting of 15 closely related serine proteases, KLK1-15 [23]. The KLKs are expressed throughout the human body and perform various physiological roles. Whereas KLK1 participate in regulation of blood pressure via the generation of bradykinin [24], KLK2, 3, 4, 5, and 14 are, for example, expressed in the prostate and are thought to be responsible for regulating semen liquefaction through hydrolysis of semenogelin [25,26]. Desquamation of the skin is mainly regulated by KLK5, 7, and 14, which are expressed in the outer layer of the epidermis and cleave cellular adhesion proteins [27]. Besides, KLK6 and KLK8 play important roles in neuronal plasticity in the central nervous system [28].

The human KLK genes are located on chromosome 19q13.3-13.4, forming the largest contiguous cluster of proteases within the human genome (**Figure 1**) [23]. Here, the 15 KLK genes are tightly clustered in a tandem array without interruption by non-KLK genes. With the exception of KLK2 and KLK3, the direction of gene transcription is from telomere to centromere in all KLKs. KLK genes range from 4–10 kb and all have 5 coding exons and 4 intervening introns with a conserved intron phase pattern (I, II, I, 0). Coding-exon lengths are similar among KLK genes, but intron lengths vary from each other. Coding exon 2, 3 and 5 harbors the conserved histidine (H), aspartic acid (D), and serine (S) codons of the catalytic residues. Most KLK genes also contain 1 or 2 non-coding exons in the 5′ untranslated region (UTR). KLK proteins are secreted serine proteases. They are synthesized as pre-pro-enzymes consisting of a signal sequence (pre-) that leads them to the endoplasmic reticulum for secretion, a propeptide (pro-) that keeps them as inactive precursors (zymogens) and a serine-protease domain responsible for the catalytic activity. The majority of these enzymes display trypsin-like activity, whereas three enzymes (KLK3, 7, and 9) show chymotrypsin-like activity [27,29-31].

Alternatively processed mRNA transcripts of KLK genes are also commonly seen. More than 80 KLK mRNA isoforms have been described. In most cases, alternative splicing occurs in coding regions, but some also within the 5´ UTR [32]. Some reports suggest that KLK mRNA isoforms are relevant for neoplastic progression: three KLK4 [33], three KLK5 [34-36], one KLK7 [34] and two KLK8 [37] alternatively spliced transcripts are found in higher levels in ovarian tumors and/or cell lines compared with normal ovaries.

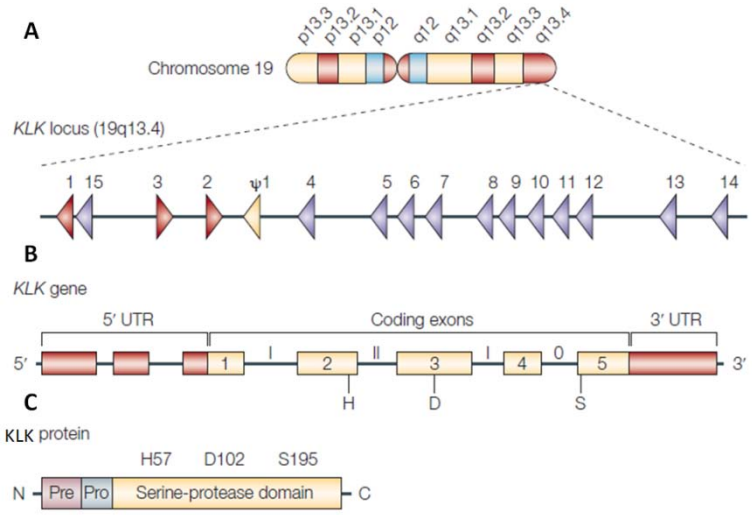

Figure 1. **Kallikrein-related peptidases: chromosomal locus, gene and protein characteristics**

A. The *KLK* locus on chromosome 19q13.3-13.4. The 'classical' *KLK* genes (*KLK1*, *2*, and *3*): red arrowheads; KLK4-15: purple arrowheads; the *ψKLK1*: yellow arrowhead. **B**. *KLK* genes (4–10 kb in length) consist of 5 coding exons (yellow boxes) and 4 intervening introns (I, II, I, 0). **C**. KLK proteins are synthesized as pre-pro-enzymes containing a signal sequence (pre-), a propeptide (pro-) and a serine-protease domain responsible for the catalytic activity. The figure is taken and slightly modified from [**23**].

KLKs are reported to be expressed in a variety of normal tissues at both mRNA and protein level. **Figure 2** and **Figure 3** summarize the mRNA and protein levels of the 15 KLKs in normal tissues [31,38-54].

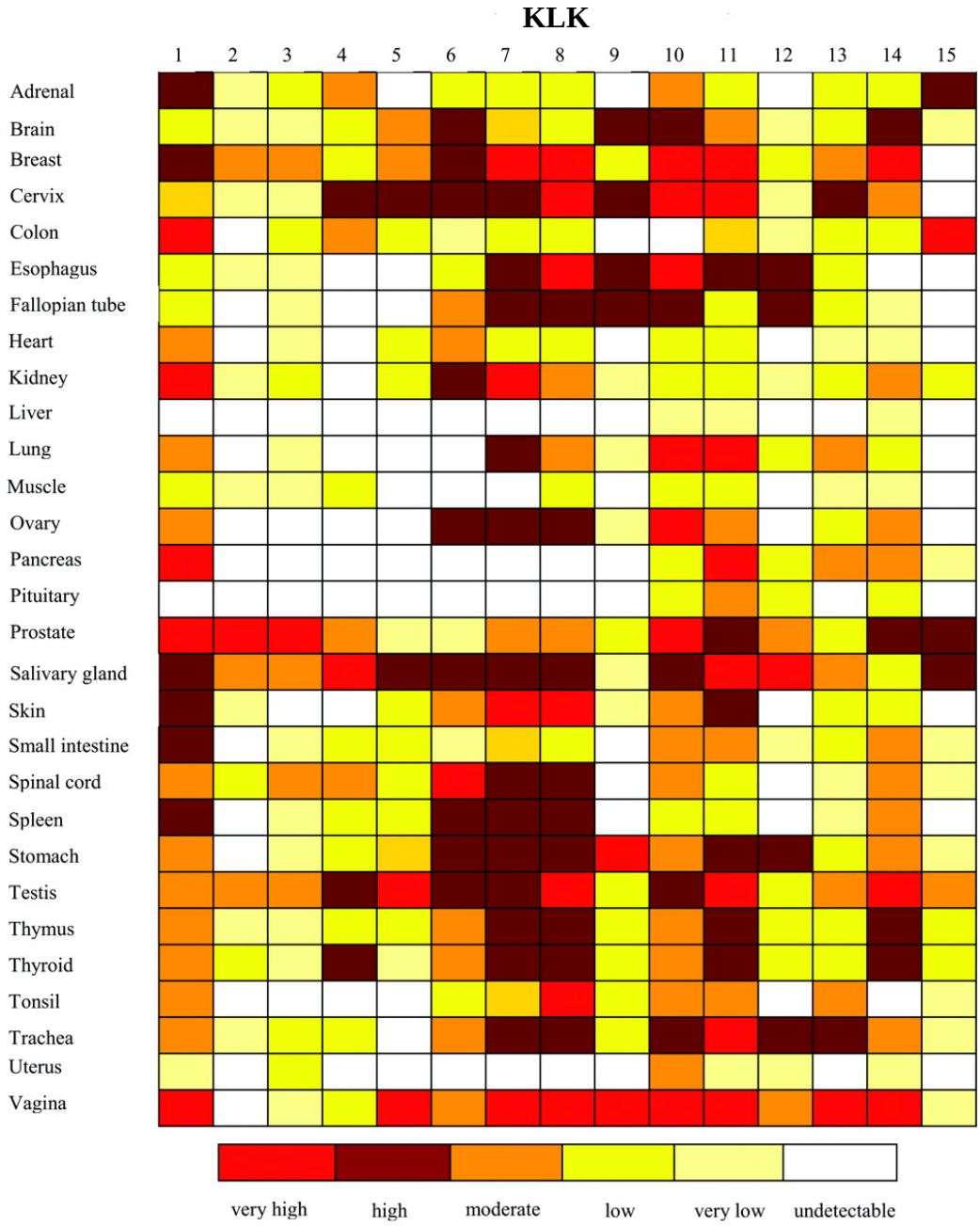

Figure 2. Expression of KLK mRNA in normal tissues

RT-PCR was performed to compare KLK levels in normal tissues. The abundance was classified by semiquantitative scoring, based on visual comparison of band intensities of ethidium bromide–stained agarose gels. 1 to 15: KLK1 to KLK15. The figure is taken and slightly modified from [**38**].

As mentioned above, all tissue KLK proteins are synthesized as enzymatically inactive forms. The inactive precursors are activated by proteolytic removal of the pro-peptide by - in most cases trypsin-like - proteases [55]. For example, KLK2, 6, and 13 are auto-activated [56-58]. On the contrary, some KLKs are activated by other KLKs: pro-KLK3 is activated by KLK2, 4, and 15 [59,60]; pro-KLK7 is activated by

KLK5 and other serine proteases [61-63]. After activation, KLKs are regulated by endogenous serine protease inhibitors such as the so-called serpins α2-antiplasmin, antithrombin III and PAI-1 (plasminogen activator inhibitor type–1), or Kazal-type inhibitors such as LEKTI and SPINK6, or α2-macroglobulin, which all form complexes with KLKs [40,57,64-69]. On the other hand, internal cleavage and subsequent degradation are also inactivation ways for several KLKs [57,58,67].

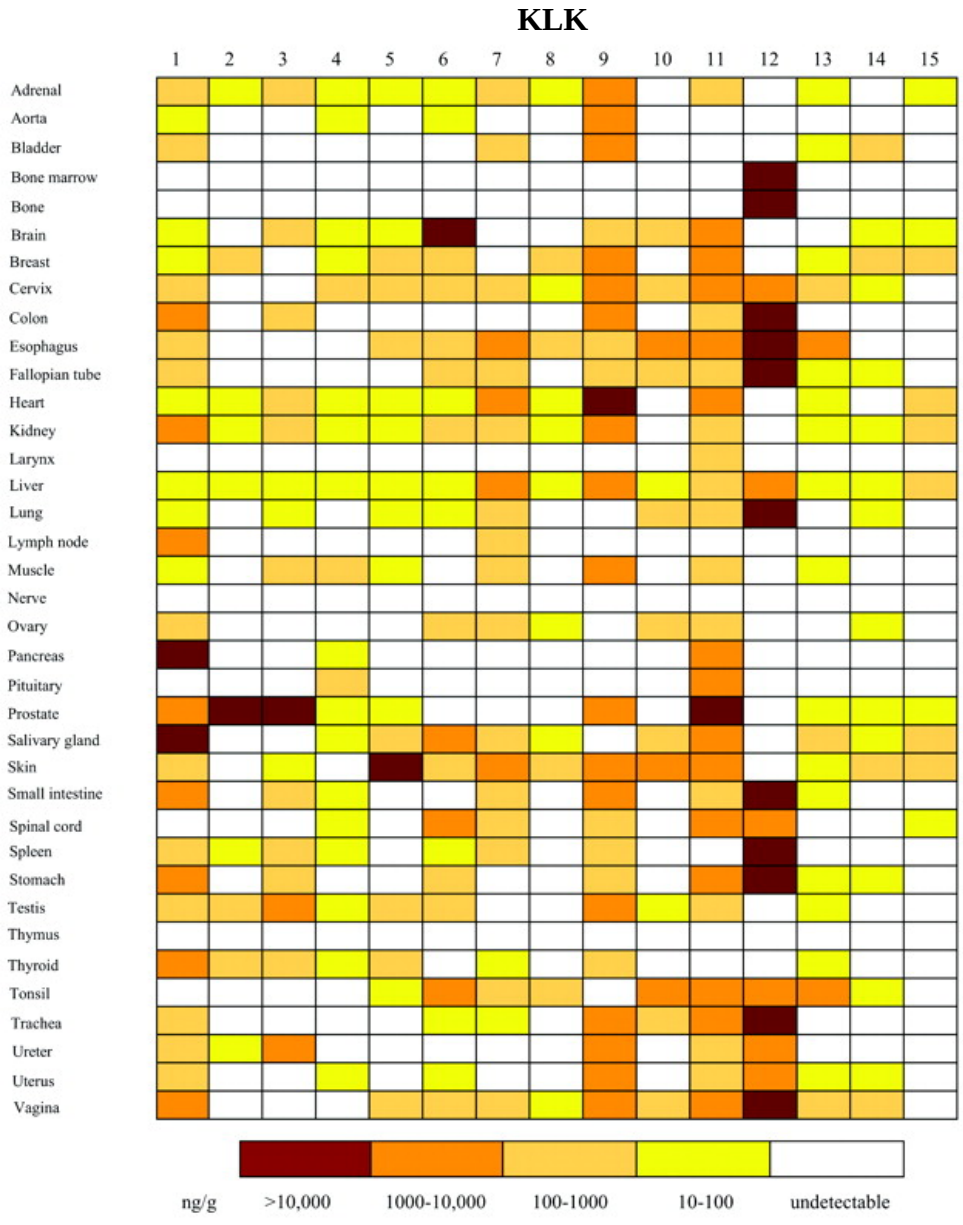

Figure 3. KLK protein concentrations in normal tissue extracts
KLK protein concentrations were measured by KLK-specific ELISAs and are indicated by color codes according to the scale on the bottom. 1 to 15: KLK1 to KLK15. The figure is taken and slightly modified from [**38**].

Different KLKs have also been observed to be co-expressed in the same tissues, including steroid hormone-relevant tissues like prostate, breast, testis, and ovary. This parallel expression pattern suggests that KLKs may be involved in enzyme cascade reactions [70]. KLK gene expression is regulated by several factors, *e.g.* steroid hormones, which influence some signaling pathways [32,71]. KLK2 and KLK3 represent classical androgen-regulated genes [72]. In contrast, KLK1, 5, 6, and 10 are more sensitive to estrogen [73-76]. Expression of other KLK genes is influenced by hormones depending on the tissue types [77-79].

1.3 Tissue kallikrein-related peptidases in cancer

More and more reports indicate that KLKs are dysregulated in cancers, especially in steroid-hormone-related cancers (prostate, breast and testicular tumors). **Tables 2-4** summarize expression and clinical relevance of KLKs in these three cancer types. KLKs are also found dysregulated in other tumor types, including acute lymphoblastic leukemia, colon cancer, lung adenocarinoma, head and neck squamous cell carcinoma, and pancreatic cancer [80-84].

In prostate cancer (**Table 2**), KLK2, 3, 5, 6, 10, and 13 show downregulation compared with normal tissue, while KLK4, 11, and 15 are upregulated in prostate cancer tissue. In the serum of prostate cancer patients, elevated KLK3 and KLK11 levels have been detected. Besides, KLK3, also known as PSA (prostate specific antigen), is widely used as a diagnostic marker and for monitoring of therapeutic efficiency in prostate cancer (PSA levels in the serum) [67]. KLK2, similar to KLK3, is also used as a diagnostic marker [67].

In breast cancer (**Table 3**), KLK3, 6, 8, 10, 12, and 13 are found downregulation. The expressions of KLK5 and KLK14 are different from other KLKs. KLK5 and KLK14 expressions are decreased in breast cancer tissue, but show upregulation in the serum of breast cancer patients. This inconsistency between tissue and serum levels could be due to the fact that more KLK proteins are released into serum during carcinogenesis and angiogenesis. In addition to prostate cancer, KLK3 may represent a diagnostic and prognostic marker for breast cancer [101].

Table 2. Clinical relevance of KLKs in prostate cancer

KLK	Expression	Clinical relevance
2	↓in malignant tumors [85]	Diagnosis [67]
3	↓in malignant tumors [85,86]	Population screening, diagnosis, prognosis, monitoring [67]
4	↑in malignant tumors [87]	ND
5	↓in malignant tumors [88]	Prognosis [88]
6	↓in malignant tumors [89]	ND
10	↓in malignant tumors [89,90]	ND
11	↑in malignant tumors [91]; ↑in serum of cancer patients [92]	Diagnosis [92,93]; prognosis [91]
13	↓in malignant tumors [89]	ND
14	↓in malignant tumors [94]; ↑in malignant tumors [95]	Prognosis [95]
15	↑in malignant tumors [96,97]	Prognosis [96,97]

↑ increase; ↓ decrease; ND not determined. The table is taken and slightly modified from [**23**].

Table 3. Clinical relevance of KLKs in breast cancer

KLK	Expression	Clinical relevance
3	↓in malignant tumors [98]	Prognosis [99-101]; predictive of response to tamoxifen [101]
5	↓in malignant tumors [102]; ↑in serum of cancer patients [76]	Diagnosis [76]; prognosis [103]
6	↓in malignant tumors [102]	ND
7	ND	Prognosis [104]
8	↓in malignant tumors [102]	ND
9	ND	Prognosis [105]
10	↓in malignant tumors and cell lines [102,106,107]	Predictive of response to tamoxifen [108]
12	↓in malignant tumors [109]	ND
13	↓in malignant tumors and cell lines [52]	Prognosis [110]
14	↓in malignant tumors and cell lines [94]; ↑in serum of cancer patients [111]	Diagnosis [111]; Prognosis [112]
15	ND	Prognosis [113]

↑ increase; ↓ decrease; ND not determined. The table is taken and slightly modified from [**23**].

In testicular cancer (**Table 4**), KLK5 expression is increased in tumor tissue and overexpression of KLK5 is associated with favorable prognosis [114]. KLK10 and KLK14 show downregulation in tumor tissue [94,115].

Table 4. Clinical relevance of KLKs in testicular cancer

KLK	Expression	Clinical relevance
5	↑in malignant tumors [114]	Prognosis [114]
10	↓in malignant tumors [115]	ND
14	↓in malignant tumors [94]	ND

↑ increase; ↓ decrease; ND not determined. The table is taken and slightly modified from [**23**].

As shown in the tables above, KLK expression is often correlated with patient prognosis. In most cases, high KLK expression is linked with poor prognosis, but some reports demonstrate that high levels of KLKs can be favorable prognostic indicators as well. These results suggest that KLKs may be involved in tumorigenesis/progression and may have dual roles as stimulatory or inhibitory factors depending on the specific tumor type and/or tumor microenvironment. Although the mechanisms are yet not fully understood, the studies indicate that KLK-mediated pericellular proteolysis is involved in many steps of cancer development, including cell growth and apoptosis, angiogenesis, invasion, and metastasis.

1.3.1 KLKs and tumor growth

By modulating the availability and activity of latent growth factors, KLKs can regulate cancer cell proliferation. For example, KLK1, 2, and 3 collectively degrade IGFBP (insulin-like growth factor binding protein) 2, 3, 4, and 5. This leads to the release of the growth factors (IGF1 and IGF2), which then bind to the IGF1-receptor to stimulate cell growth [116-119]. KLK2 and KLK4 may regulate the uPA (urokinase-type plasminogen activator) system by activating uPA and inactivating PAI-1 (plasminogen activator inhibitor type 1). The uPA system converts plaminogen to plasmin, leading to extracellular matrix (ECM) degradation and growth factor release [59,120,121]. KLKs can also act as growth factors themselves through activation of PAR (protease-activated receptor) signaling [122,123]. On the other hand, KLKs may also inhibit cell growth as exemplified by KLK3, which can activate

latent transforming growth factor-β, which acts as a suppressor of growth and inducer of apoptosis in many different cell-types [124]. In addition, KLK3 stimulates ROS (reactive oxygen species) production in prostate cancer cells, which can cause DNA damage and apoptosis [125]. KLK10 is also considered as a tumor suppressor due to its downregulation in several cancers (breast, prostate and testicular cancer) [90,106,107,115].

1.3.2 KLKs and angiogenesis

Angiogenesis is the formation of new blood vessels in tissue. KLKs may enhance this process by facilitating endothelial cell proliferation and capillary tube formation through ECM degradation. KLK2, 3, 6, and 7 directly degrade ECM proteins [57,126-130]. KLK1 activates pro-MMP2 and pro-MMP9, which subsequently degrade collagen IV and other components of the basement membrane [131-133]. Another mechanism is the above mentioned activation of uPA system, which further promotes ECM degradation through plasmin, VEGF (vascular endothelial growth factor) and MMPs [59,120,121]. KLKs may also induce endothelial cell proliferation by PAR signaling [134]. Conversely, KLKs can inhibit the angiogenesis process. KLK3, 6, and 13 can release angiostatin-like fragments from plasminogen, which are potent inhibitors of endothelial cell proliferation and angiogenesis [58,135,136]. KLK3 can also block fibroblast growth factor 2 (FGF2) and/or VEGF-stimulated endothelial cell proliferation, migration and invasion [137,138].

1.3.3 KLKs and metastasis

As mentioned above, KLKs can degrade the ECM, which facilitates tumor cell detachment and invasion. KLK3 may release TGFβ from its latent complex and therefore promotes epithelial-to-mesenchymal transition (EMT), which is a prerequisite for tumor cells to invade and metastasize [111]. EMT is the process of extensive changes in the expression of adhesion molecules, including the loss of E-cadherin and expression of N-cadherin. On the contrary, one study showed that mice treated with KLK3 had strikingly lower numbers of lung metastases compared with controls, suggesting that KLK3 inhibited the metastatic process [137].

1.4 Kallikrein-related peptidases in ovarian cancer

Most KLKs, *i.e.* KLK2-8, 10, 11, 13, and 15, show upregulation in ovarian cancer as summarized in **Table 5**. Increased levels of KLK5, 6, 8, 10, 11, and 14 proteins have been also confirmed in serum of ovarian cancer patients. Concerning the patients' prognosis, KLK expression has different effects. Elevated KLK4-7, 10, and 15 expression seems to be correlated with poor prognosis, whereas high levels of KLK8, 9, 13, and 14 are associated with favorable prognosis.

Table 5. Clinical relevance of KLKs in ovarian cancer

KLK	Expression	Clinical relevance
2,3	↑in malignant tumors [139]	ND
4	↑in malignant tumors and cell lines [33,140]	Prognosis [140]
5	↑in malignant tumors and cell lines [34,76,141-143]; ↑in serum and ascites fluid in cancer patients [142]	Diagnosis [142]; prognosis [141,144]
6	↑ in malignant tumors and tumors of low malignant potential [137,139,142, 143,145-148]; ↑in serum of cancer patients [58,149]	Diagnosis [149,150]; prognosis [150,151]; monitoring [149,150]
7	↑in malignant tumors and cell lines [34,139,142,143,152]	Prognosis [153]
8	↑in malignant tumors [37,139,142,143,154-156]; ↑in serum of cancer patients [155,157]	Diagnosis [155]; prognosis [37,155,156]; monitoring [155]
9	ND	Prognosis [158]
10	↑in malignant tumors [139,142,146,147,159]; ↑in serum of cancer patients [160,161]	Diagnosis [160,161]; prognosis [159,161]; monitoring [160]
11	↑in malignant tumors [139,142,162]; ↑in serum of cancer patients [92]	Diagnosis [92]; prognosis [162-164]
13	↑in malignant tumors [139,165]	Prognosis [166]
14	↑in malignant tumors [94]; ↓in malignant tumors [111,142]; ↑ in serum of cancer patients [111]	Diagnosis [111]; prognosis [167]
15	↑in malignant tumors [168]	Prognosis [168]

↑ increase; ↓ decrease; ND not determined. The table is taken and slightly modified from [**23**].

In some cases, somewhat contradictory results have been published. High mRNA levels of KLK11 are associated with poor prognosis, but overexpression of the KLK11 protein indicates favorable prognosis in ovarian cancer [162,163]. Interestingly, studies also demonstrate that KLK6 and KLK10 increase the sensitivity and specificity of CA125 in detecting early stage (I/II) ovarian cancer [150,161].

In addition to the reports of the effects of single KLKs, accumulating evidence suggests that certain tissue KLKs, especially KLK4-7, are part of an enzymatic cascade pathway which is activated in ovarian cancer [169-173].

Prezas and co-workers [170] stably transfected OV-MZ-6 ovarian cancer cells simultaneously with plasmids expressing KLK4, 5, 6, and 7, respectively. They found that these cells, expressing and secreting all four KLKs, showed a similar proliferative capacity as vector control cells, but displayed a significantly increased invasive behavior in an *in vitro* Matrigel invasion assay. The cancer cells were also inoculated into the peritoneum of nude mice. Simultaneous expression of KLK4-7 led to a significant increase in tumor burden compared with the vector control cell line. These results suggested that tumor-associated overexpression of tissue KLKs contributed to ovarian cancer progression.

In a follow-up study, Loessner *et al.* [171] have demonstrated that overexpression of KLK4-7 in OV-MZ-6 ovarian cancer cells decreased integrin expression, with consequently less cell-matrix attachment and resistance to paclitaxel. They indicated that these KLKs might affect proteolytical cascades promoting progression and malignant phenotypes of ovarian cancer. Furthermore, the authors embedded KLK4-7-cotransfected OV-MZ-6 cells in a bioengineered three-dimensional (3D) microenvironment to analyze the spheroid growth. Larger spheroids were formed in KLK4-7-overexpressing cells than control cells in 3D. In line with the previous report [170], overexpression of KLK4-7 promoted tumor growth after 4 weeks and intra-peritoneal spread after 8 weeks in a spheroid-based animal model, indicating that KLK4-7 overexpression induced early-phase tumor growth and late-phase metastatic spread in the xenograft mouse model [172].

In addition, Shahinian and co-workers [173] have investigated how KLK4-7 expression affected the secreted proteome (secretome) and proteolytic profile (degradome) of ovarian cancer cells. Interestingly, KLK4-7 significantly regulated the abundance of proteins participated in cell-cell communication. KLK4-7

overexpressing OV-MZ-6 cells showed elevated TGFβ-1 signaling, including increased levels of neural cell adhesion molecule L1 (L1CAM). Increased levels of TGFβ-1 and L1CAM upon expression of KLK4-7 were also confirmed *in vivo* by an ovarian cancer xenograft model. By degradome analysis, KLK4-7 expression was found mainly affecting cleavage sites C-terminal to arginine, corresponding to KLK 4, 5, and 6. The authors suggested that KLK4-7 displayed a strong, yet non-degrading impact on the secretome, with an association between KLKs and TGFβ-1 signaling in the tumor cells.

In summary, the reports investigating ovarian cancer cells overexpressing KLK4-7 [170-173] indicate that KLK4-7 overexpression increases the malignancy of ovarian cancer cells, and KLK4-7 seem to have a regulatory but not only degradative role in OV-MZ-6 cells. However, whether KLK4-7 regulate expression of other genes and which genes and pathways are regulated has not been elucidated yet. Therefore, the current project focuses on analyzing these questions.

2 Aims of the study

KLKs, as serine proteases, are supposed to have a degradative role in ovarian cancer, but previous reports suggest that KLK4-7 may also display regulatory functions in ovarian cancer cells. Therefore, this project aimed at, on one hand, analyzing whether KLK4-7 overexpression affects expression of other cancer-related genes in OV-MZ-6 ovarian cancer cells and, on the other, characterizing the effects of overexpression of individual KLKs (KLK4, 5, 6, or 7) on gene regulation in OV-MZ-6 cells. To achieve these aims, the following investigations were performed:

1) Three different PCR array analyses were conducted to screen for differentially regulated genes in OV-KLK4-7 cells compared with OV-VC cells. Each PCR array contains 84 genes which are related to one of the following pathways: TGFβ-signaling, PAR-signaling, and epithelial-to-mesenchymal transition (EMT). The PCR arrays have been selected based on previous reports which indicated an involvement of KLKs in these tumor-relevant pathways. In addition to the obtained PCR array data, data from genome-wide RNA microarray and proteomic analyses were also combined to select genes for further validation.

2) qPCR, Western blot, immunofluorescence, and immunohistochemistry analyses were performed to validate the findings from the PCR array screening on both mRNA and protein level.

3) Expression of differentially regulated genes/proteins in OV-KLK4-7 *vs.* OV-VC cells, was also characterized in OV-KLK4, OV-KLK5, OV-KLK6, and OV-KLK7 cells by qPCR and Western blot to analyze effects of individual KLKs on gene regulation in OV-MZ-6 cells. This analysis could unveil which KLK exerts the strongest impact on gene regulation and may lead to a deeper understanding of the mechanism by which KLK4-7 overexpression regulates the expression of other genes in OV-MZ-6 cells.

4) Cell migration assay was performed to analyze the effects of overexpression of KLKs on ovarian cancer cell behavior. Furthermore, immunohistochemical methods were established enabling expression analysis of the differentially expressed proteins in tumor tissue from patients afflicted with ovarian cancer.

3 Materials and Methods

3.1 Cell culture

3.1.1 Thawing of OV-MZ-6 cells

OV-MZ-6 cells were thawed quickly and transferred to a 15 ml Falcon tube with 5 ml complete medium (Dulbecco's Modified Eagle Medium [DMEM] with 10% fetal calf serum [FCS] and 10 mM HEPES). Thawed cells were centrifuged at 1,300 rpm for 3 min and the medium was discarded. Fresh medium was added into the Falcon tube and cells were resuspended. After that the centrifuge step was repeated again. Then, the cells were resuspended in complete medium and cultured in flasks. After attachment of cells, *i.e.* after 4 h, the medium was replaced by fresh one.

3.1.2 Cultivation of OV-MZ-6 cells

OV-MZ-6 cells were cultured in a 75 cm^2 flask in the incubator at 5% CO_2 (v/v), 95% humidity and 37°C in complete medium (see **3.1.1**). For cell passage, the cells were detached with EDTA/PBS (1% w/v) for 5 min and washed with 5 ml PBS. After centrifugation at 1,300 rpm for 3 min, the medium was discarded. Cells were resuspended in complete medium and transferred into a new flask.

3.1.3 Detection of mycoplasma contamination

Mycoplasma contamination of cultured cells was regularly analyzed using mycoplasma-specific PCR primers (3′ primer: 5′-GCG GTG TGT ACA AGA CCC GA- 3′; 5′ primer: 5′-CGC CTG AGT AGT ACG TTC GC- 3′). For this, DNA was extracted from OV-MZ-6 cells using the 'DNA High Pure PCR Template Preparation Kit' (Roche Diagnostics) according to the manufacturers' instructions. Mycoplasma PCR was performed using master mix **A** and according to PCR program **B**. Finally, the PCR products were analyzed by electrophoresis (100V, 30 min) in 1% agarose with ethidium bromide (0.25 µg/ml).

A) PCR master mix:

- 10x PCR reaction buffer 2.5 µl
- dNTPs (2.5 mM) 2.5 µl
- Primer 5′ (20 pM) 1.5 µl
- Primer 3′ (20 pM) 1.5 µl
- Taq polymerase (5 U/µl) 0.5 µl
- H_2O 15.5 µl
- Extracted DNA 1.0 µl

(Total volume 25µl)

B) PCR program:

- Step 1: 5 min at 94°C
- Step 2: 30 sec at 94°C
- Step 3: 1 min at 60°C
- Step 4: 30 sec at 72°C
- Step 5: go to step 2 (30 times)
- Step 6: 5 min at 72°C

3.1.4 Treatment of mycoplasma infection

In most cases, cells were free of mycoplasma as shown in **Figure 4**. If cells were positive for mycoplasma, they were treated with Myco-3 (AppliChem). Most mycoplasma species are sensitive to Myco-3, including *A. laidlawii*, *M. orale*, *M. hyorhinis*, *M. fermentans* and *M. arginini*, which are commonly seen in mycoplasma contamination in cell culture. Myco-3 was added to the complete medium at 1 µg/ml for 14 d according to the manufacturers' instructions.

3.1.5 Freezing of OV-MZ-6 cells

OV-MZ-6 cells were detached and $1x10^6$ cells were resuspended in 1 ml FCS containing 10% dimethylsulfoxid (DMSO). The cells were frozen at -80°C in cryogen micro tubes (Nalgene) for up to 24 h and transferred into liquid nitrogen at -196°C.

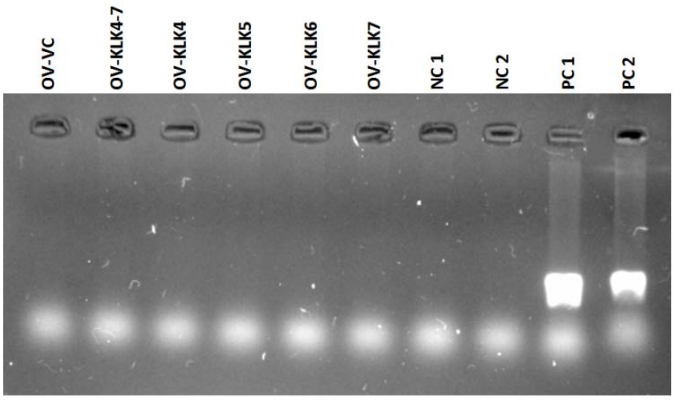

Figure 4. Mycoplasma assay results of six OV-MZ-6 cell lines (OV-VC, OV-KLK4-7, OV-KLK4, OV-KLK5, OV-KLK6, OV-KLK7)

PCR was performed using the DNA samples extracted from OV-MZ-6 cells. PCR products were analyzed by electrophoresis (100V, 30 min) in 1% agarose with ethidium bromide (0.25 μg/ml). Positive control samples (PC1, PC2) showed clear mycoplasma PCR products. The OV-MZ-6 cell lines and negative control samples (NC1, NC2) were all mycoplasma negative.

3.1.6 Culturing of OV-MZ-6 cells in a 3D model

Irgacure 2959 (photo-initiator) was dissolved at 0.6 mg/ml in PBS at 70°C and mixed by vortexing until it was completely dissolved. Then the Irgacure 2959 solution was added to gelMA (gelatin methacryloyl) foam to completely immerse the foam (gelMA concentration: 10% w/v). The final solution was stored at 4°C overnight. Cells were resuspended in PBS and mixed with gelMA solution (final density: $3x10^5$ cells/ml). The gelMA/cell suspension was pipetted into a Teflon mould (**Figure 5A**) and was cured in the centre of the ultraviolet lamp (UVP, 254 nm) for 15 min. After that, the gel was cut into pieces using a scalpel and a cutting guide (**Figure 5B**). Finally, gel pieces containing cells were transferred to 24-well plates with complete medium for 3D culturing [174].

3.2 Quantitative PCR analysis

3.2.1 Isolation of RNA

RNA was isolated from OV-MZ-6 cells using the RNeasy Mini kit (Qiagen) and the QIAcube fully automated spin column system (Qiagen). An on-column DNase (Qiagen) treatment was performed as well. The recommended protocol of the manufacturer was exactly followed. ND1000 (NanoDrop) was used to measure the

concentration and purity of the isolated RNA.

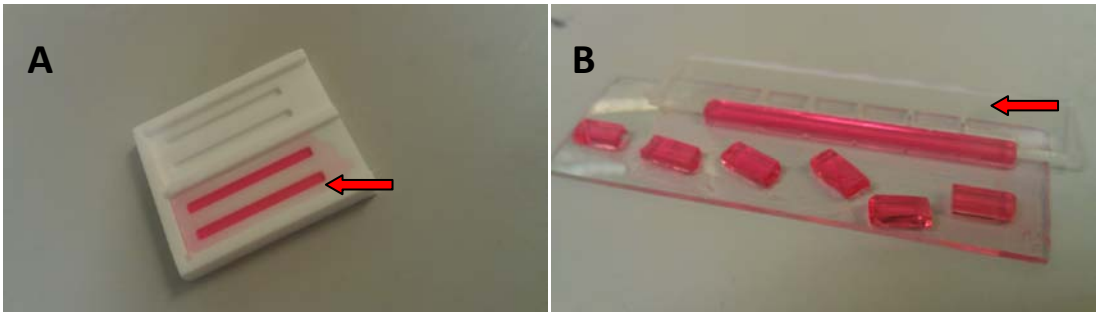

Figure 5. Teflon mould and cutting guide used in 3D culture
A: The Teflon mould was filled with the gelMA/cell suspension (two strips, indicated by the arrow). Red dye was added for visualization. A slight excess was to avoid bubbles inside gels. B: Cutting guide (indicated by the arrow) was used for cutting even-sized gel pieces.

3.2.2 Reverse transcription

Reverse transcription was performed with the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). The cDNA synthesis was done as follows:

- Isolated RNA 1.0 µg
- Random hexamer primers (50 ng/µl) 1.0 µl
- H_2O x µl

Total 10.0 µl

The RNA/primers mixture was incubated at 65°C for 5 min. Then the following master mix was added at 10.0 µl per well to the mixture:

- DTT (0.1 M) 1.0 µl
- dNTPs (10 mM) 2.0 µl
- RNase inhibitor (40 U/µl) 1.0 µl
- AMV transcriptase (15 U/µl) 1.0 µl
- H_2O 1.0 µl
- Buffer (5x) 4.0 µl

The PCR reaction mixture was assembled on ice and the PCR program was used as follows:

Step 1: 10 min at 25°C

Step 2: 45 min at 60°C

Step 3: 5 min at 85°C

The final cDNA was diluted 1:10 with H_2O and stored at -20°C.

3.2.3 qPCR analysis using Universal ProbeLibray probes

Universal ProbeLibray (UPL) probes (Roche) are short hydrolysis probes with only 8 - 9 nucleotides. The Stratagene Mx3005P qPCR System (Agilent Technologies) was used for qPCR analysis. 25 ng of cDNA was utilized per well. The qPCR master mix was comducted as follows:

- 2x Brilliant III qPCR master mix with low ROX 10.0 µl (Agilent Technologies)
- UPL Probe (10 µM) 0.4µl
- Primer forward (20 µM) 0.4 µl
- Primer reverse (20 µM) 0.4 µl
- H_2O 3.8 µl
- cDNA 5.0 µl

Total 20µl.

All qPCR reactions were conducted in triplicates. The samples were added into 96-well plates which were further sealed by an optical strip and centrifuged for 1 min at 1,500 rpm to remove bubbles. The following qPCR program was performed:

- Step 1: 3 min at 95°C
- Step 2: 15 sec at 95°C
- Step 3: 20 sec at 60°C
- Step 4: go to step 2 (40 times).

3.3 RT² Profiler PCR array analysis

The SABiosciences RT² Profiler PCR arrays are designed to analyze a panel of genes related to a disease or a biological pathway. The 96-well plates contain primers for 84 pathway- or disease-focused genes and five housekeeping genes. In addition, one well contains a genomic DNA control, three wells contain reverse-transcription controls, and three wells contain positive PCR controls. The RT² First Strand Kit was used for

cDNA synthesis. RT² SYBR Green Master Mix and SYBR Green-optimized primers were used for the qPCR according to the manufacturers' instructions.

3.3.1 Human TGFβ RT² Profiler PCR array

The Human TGFβ Signaling RT² Profiler PCR Array consists of 84 key genes involved in TGFβ signaling. The array includes members of the TGFβ superfamily of cytokines and their receptors such as TGFB1-3 and BMP1-7. In addition, SMAD and SMAD target genes such as ATF4 and ID1 are present on the array. Other genes including adhesion and extracellular molecules are present as well (such as COL1A1, COL1A2). Finally, some of the genes involved in downstream cellular processes are also included (CDKN1A, DCN). At first 1.5 μg RNA was converted into cDNA using the First Strand Kit (Qiagen) as described above (**3.2.2**). Next, the cDNA was mixed with SYBR Green master mix. Then, this mixture was aliquoted into the wells of the RT² Profiler PCR array (20 μl per well). PCR was performed according to the program mentioned in **3.2.3**.

3.3.2 Human PAR RT² Profiler PCR array

The Human Protease-Activated Receptor Signaling RT² Profiler PCR array contains 84 key genes involved in the activation and response of protease-activated receptors (PARs). The PAR family (PAR1-4) is a class of receptors which are activated by proteolytic cleavage of the extracellular domain. Thrombin (F2) activates PAR1, 2, and 4, while trypsin activates PAR3. PAR signaling interacts with other cellular receptors, such as EPCR and S1PR3. PAR signaling pathways have been identified in various cell types, involved in biological processes such as proliferation, adhesion, and migration. The results of this array could suggest which PARs are involved in the interested model. The procedure was carried out as described for the TGFβ RT² Profiler Array with 1.5 μg of RNA per array.

3.3.3 Human EMT RT² Profiler PCR array

The Human Epithelial to Mesenchymal Transition (EMT) RT² Profiler PCR array includes 84 key genes that either change expression during this process or regulate those gene expressions. EMT is an important process involved in tumor metastasis. During EMT process, epithelial cells lose their cellular polarity and break the intercellular tight junctions to become mesenchymal cells. As such, the array consists of cell surface receptor, extracellular matrix, and cytoskeletal genes (such as MSN,

KRT7, COL5A2) regulating cell adhesion, migration, and morphogenesis. Genes involved in cell differentiation, proliferation, and signal transduction are also present (such as EGFR, FGFBP1, IGFBP4). The procedure was carried out as described for the TGFβ RT² Profiler array with 1.5 µg of RNA per array.

3.4 Western blot analysis

3.4.1 Preparation of cell protein samples

$3x10^6$ cells were detached using 1% EDTA and washed with PBS. The cell pellet was lysed in 100 µl lysis buffer (TBS; 1% Triton X-100; Complete proteases inhibitor cocktail tablet with EDTA [Roche]). After incubation on ice for 1 h, lysates were centrifuged at 14,000 g for 10 min and the supernatant containing proteins was frozen at -20°C. The total protein content was measured with the BCA Protein Assay Kit (Thermo Fisher). Loading samples for SDS-PAGE were prepared as follows and boiled at 95°C for 5 min:
30 µl protein sample (40 µg) + 10 µl SDS-PAGE loading buffer (4X);
10 µl PageRuler Plus Prestained Protein Ladder (Thermo Fisher) + 20 µl TBST (TBS; 1% Tween-20) + 10 µl SDS-PAGE loading buffer (4X).

3.4.2 Preparation of SDS gels

The 12% PAA (polyacrylamide) seperating gel was prepared freshly as follows:
- H_2O: 6.5 ml;
- 1.5 M Tris HCl (pH8.8): 3.75 ml;
- 40% Acry / Bis (acrylamide-bisacrylamide, 39:1): 4.5 ml;
- 10% SDS (sodium dodecyl sulfate): 0.15 ml;
- APS (ammonium persulfate): 90 µl
- TEMED (tetramethylethylenediamine): 10 µl

The 5% PAA stacking gel was prepared as follows:
- H_2O: 17.2 ml;
- 1.5 M Tris HCl (pH6.8): 7.2 ml;
- 40% Acry / Bis (acrylamide-bisacrylamide, 39:1): 3.6 ml;
- 10% SDS: 0.28 ml;

The stacking gel solution was stored at 4°C (maximum 4 weeks). For each gel, 3 ml solution was mixed with 50 µl APS and 10 µl TEMED.

3.4.3 SDS-PAGE (polyacrylamide gel electrophoresis), blotting and protein detection

Samples were concentrated on the stacking gel at 100 V for 30 min and separated on the seperating gel at 150 V for 1 h. To transfer the proteins from the gel to a polyvinylidene difluoride (PVDF) membrane (Millipore), a tank blotting device (Bio-Rad) was used. Tank blotting, was done with glycine buffer [20% (v/v) ethanol; 25 mM Tris; 192 mM glycine] at 350 mA for 1.5 h on ice.

For detection of the protein, the PVDF membrane first was washed in TBST for 5 min at RT. Membranes were incubated for 1 h at RT in blocking buffer [TBST; 5% (w/v) milk powder]. After washing with TBST for 5 min in RT, the primary antibodies (**Table 6**) were applied overnight at 4°C with gentle shaking. The secondary antibodies conjugated with HRP were diluted 1:5,000 (1 mg/ml) in TBST with 1% (w/v) milk powder and applied for 1 h at RT with gentle shaking. Proteins were visualized using ECL chemiluminescence with V3 Western Workflow (Bio-Rad) according to the recommendations of the manufacturer.

Table 6. List of primary antibodies used

Antibody name	Company	Dilution		
		WB	IF	IHC
Rabbit anti-human anti-MSN antibody, polyclonal	HPA011135, Sigma-Aldrich, Germany (0.3 mg/ml)	1:1,000	1:100	1:500
Rabbit anti-human anti-JUNB antibody, polyclonal	HPA019149, Sigma-Aldrich, Germany (0.1 mg/ml)	1:1,000	1:150	1:100
Rabbit anti-human anti-Cytokeratin 19, monoclonal	ab52625, Abcam, Cambridge, UK (0.808 mg/ml)	1:1,000	1:300	1:400
Rabbit anti-human anti-Cytokeratin 7, monoclonal	ab68459, Abcam, Cambridge, UK (0.018 mg/ml)	1:500	1:100	1:100
Mouse anti-human anti-GAPDH, monoclonal	MAB374, Chemicon, Billerica, USA (1 mg/ml)	1:2,000	NA	NA

WB: Western blotting; IF: immunofluorescence; IHC: immunohistochemistry; NA: not applied.

3.4.4 Coomassie blue staining

After the electrophoresis (**3.4.3**), the stacking gel was cut off and the seperating gel was fixed in fixing solution (50% methanol, 10% acetic acid) overnight. Then, the gel was stained with Coomassie blue solution (0.1% Coomassie R-250, 40% ethanol, 10% acetic acid) for at least 1 h at RT and destained (40% methanol, 10% acetic acid) overnight. Finally, photos were taken with V3 Western Workflow (Bio-Rad).

3.5 Immunofluorescence (IF) analysis

The procedure has been described in detail by Loessner *et al.* [171]. In short, cells were fixed in 4% formaldehyde for 15 min at RT and then blocked in blocking buffer for 60 min. After that, blocking solution was aspirated and the primary antibodies (**Table 6**) were applied overnight at 4°C. Cells were incubated with a fluorescein-conjugated secondary antibody for 1 h at RT in the dark. Finally cells were incubated with DAPI (DNA stain) for 1 min. Slides were covered with mounting medium.

3.6 Immunohistochemistry (IHC) analysis

The paraffin-embedded ovarian cancer tissue slides were incubated at 60°C overnight and then immersed into xylol, isopropanol, 96% and 70% ethanol 5 min each for deparaffination and rehydration. After that, slides were incubated in 3% hydrogen peroxide for 20 min at RT for blocking. Then, slides were pressure-cooked in citrate buffer (citric acid 2.1 g, H_2O 1 l, pH 6.0) for 4 min. Primary antibodies (**Table 6**) were applied (120 µl for each slide) and slides were incubated for 1 h at RT in a wet chamber. Then, the secondary antibody conjugated with HRP (horseradish peroxidase) (Zytomed) was applied (120 µl for each slide) for 30 min at RT in a wet chamber. After that, 120 µl of DAB substrate per slide were added for 8 min at RT in a wet chamber. Slides were counter-stained with Haemalaun for 1 min and dehydrated with 70% and 96% ethanol, isopropanol and xylol 3 min each. Finally slides were mounted with PERTEX (Histolab).

3.7 Cell assays

3.7.1 Inhibition assay with the KLK5-specific inhibitor SFTI-FCHR Y7N14

$6x10^4$ OV-MZ-6 cells per well were seeded in triplicates on 24-well plates and were incubated in complete medium with 0, 0.5, or 2.0 µM of the KLK5 inhibitor (SFTI-FCHR Y7N14, kindly provided by Prof. Jonathan Harris, QUT, Brisbane). Cells were detached in 200, 300, 400, and 500 µl of PBS with 1% EDTA (1 h at 37°C) after 24, 48, 72, and 96 h of cultivation, respectively. Trypan blue solution (Thermo Fisher) was added to the cell suspension and the cell number was quantified by counting with a hemocytometer. The cell numbers of each cell line were compared to illustrate cell proliferation ability at each time point.

3.7.2 Migration assay

The 24-well plates were coated with the ECM substrates fibronectin and vitronectin (5 µg/ml, 300 µl per well) overnight at 37°C. Unbound ECM proteins were removed and the coated plates were blocked with 3 ml of 0.2% (2 mg/ml) bovine serum albumin for 1 h at 37°C. Then, the plates were refilled with 2 ml media. $1x10^5$ OV-MZ-6 cells per well were plated onto the prepared plates to create a confluent monolayer. The plates were incubated for approximately 6 h at 37°C, allowing cells to adhere and form a monolayer. Then the cell monolayer was scraped in a straight line to create a ‘‘scratch’’ with a p100 pipet tip. Debris was removed with PBS. Plates were filled with 2 ml serum-free medium and incubated at 37°C, 5% CO_2. Photos of the scratches were taken at 0, 12, 24, and 48 h [175].

3.8 Statistics

Data were analyzed for statistical significance using the software SPSS 22.0 (IBM, USA). For comparison of mean values, the independent-samples T-Test was used. Differences with a p-value ≤ 0.05 were considered statistically significant ($p \leq 0.05$ is marked as *, $p \leq 0.01$ as ** and $p \leq 0.001$ as ***).

4 Results

4.1 Analysis of KLK4, 5, 6, 7 mRNA expression in OV-MZ-6-derived cell lines by qPCR

Previously, several stably transfected OV-MZ-6-derived cell lines have been generated aiming at overexpressing either KLK4, 5, 6, or 7 individually or all four genes together [177]. To analyze KLK4, 5, 6, and 7 expression in the KLK-transfected cell lines, qPCR tests were performed. Vector control cells (OV-VC) express only minute amounts, if at all, of these KLK mRNAs. Still, the obtained low expression values were used as reference (relative expression was set to 1.0). OV-KLK4-7 cells showed elevated expression of KLK4, 5, 6, and 7 (57.3 ± 6.1, 603.3 ± 40.7, 41.4 ± 4.6 and 339.4 ± 39.8, respectively, **Figure 6A**). OV-KLK4 cells showed high expression of KLK4 mRNA (2,538.9 ± 321.4) but no expression of the other KLKs (**Figure 6B**). Similarly, OV-KLK5 cells showed high expression of only KLK5 mRNA (3,111.4 ± 253.2, **Figure 6C**), OV-KLK6 cells of only KLK6 mRNA (1,845.8 ± 167.9, **Figure 6D**), and OV-KLK7 cells of only KLK7 mRNA (3,691.5 ± 397.1, **Figure 6E**). Because OV-VC cells express the 4 KLKs, if at all, in very low amounts, the relative KLK mRNA values in the transfected OV-MZ-6 cells seem to be very high. However, in each case, expression of the respective KLK was found to be lower than the housekeeping gene HPRT, indicating a physiological expression rate. In summary, the qPCR analysis confirmed the expected KLK expression patterns of all six analyzed OV-MZ-6 cell lines.

4.2 Screening for regulated genes in KLK4-7-overexpressing OV-MZ-6 cells by PCR array analyses

In order to identify differentially expressed genes in OV-KLK4-7 compared to OV-VC cells, three different PCR array analyses were performed. For this, commercially available arrays (SABioscience) were used allowing analysis of 84 genes related to important tumor-asociated pathways (TGFβ, PAR and EMT). As shown in **Figure 7**, most genes showed a less than 2-fold regulation (black dots), *i.e.* expression of these genes is not (or only marginally) affected by KLK4-7 expression in these cells.

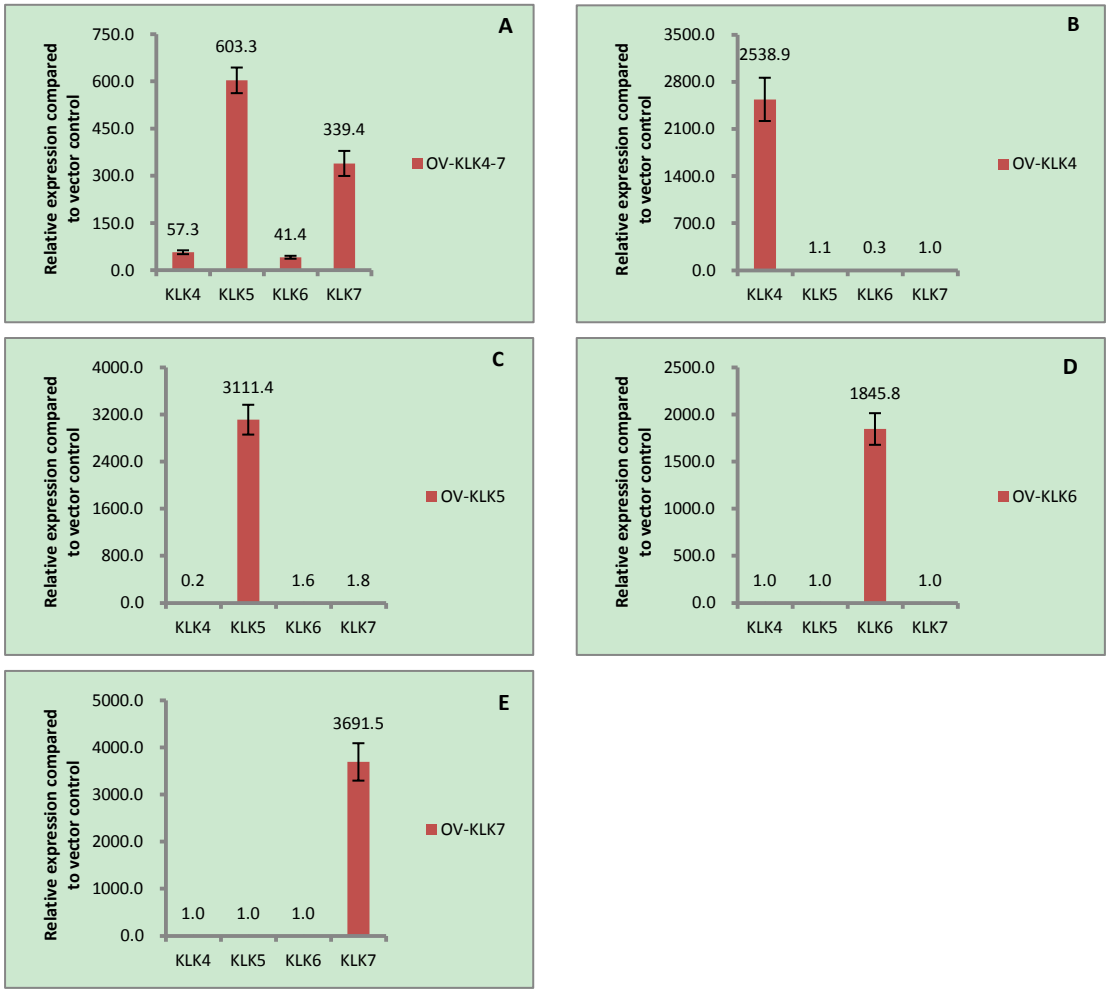

Figure 6. Analysis of KLK4, 5, 6, 7 mRNA expression in stably transfected OV-MZ-6 cells

KLK4, 5, 6, 7 mRNA expression in OV-KLK4-7 cells (A), OV-KLK4 cells (B), OV-KLK5 cells (C), OV-KLK6 cells (D), and OV-KLK7 cells (E). OV-KLK4-7 cells showed elevated expression of all four KLKs, while OV-KLK4, OV-KLK5, OV-KLK6 and OV-KLK7 displayed overexpression of a single KLK only. The values are given as relative expression values compared to the expression in OV-VC cells.

Still, some genes displayed a more than 2-fold upregulation (red dots) and some genes a more than a 2-fold downregulation (green dots) in OV-KLK4-7 compared with OV-VC cells. All in all, genes located on the EMT PCR array (**Figure 7C**) showed the most distinct fold changes, while genes analyzed by the PAR PCR array (**Figure 7B**) showed comparatively slight gene expression changes.

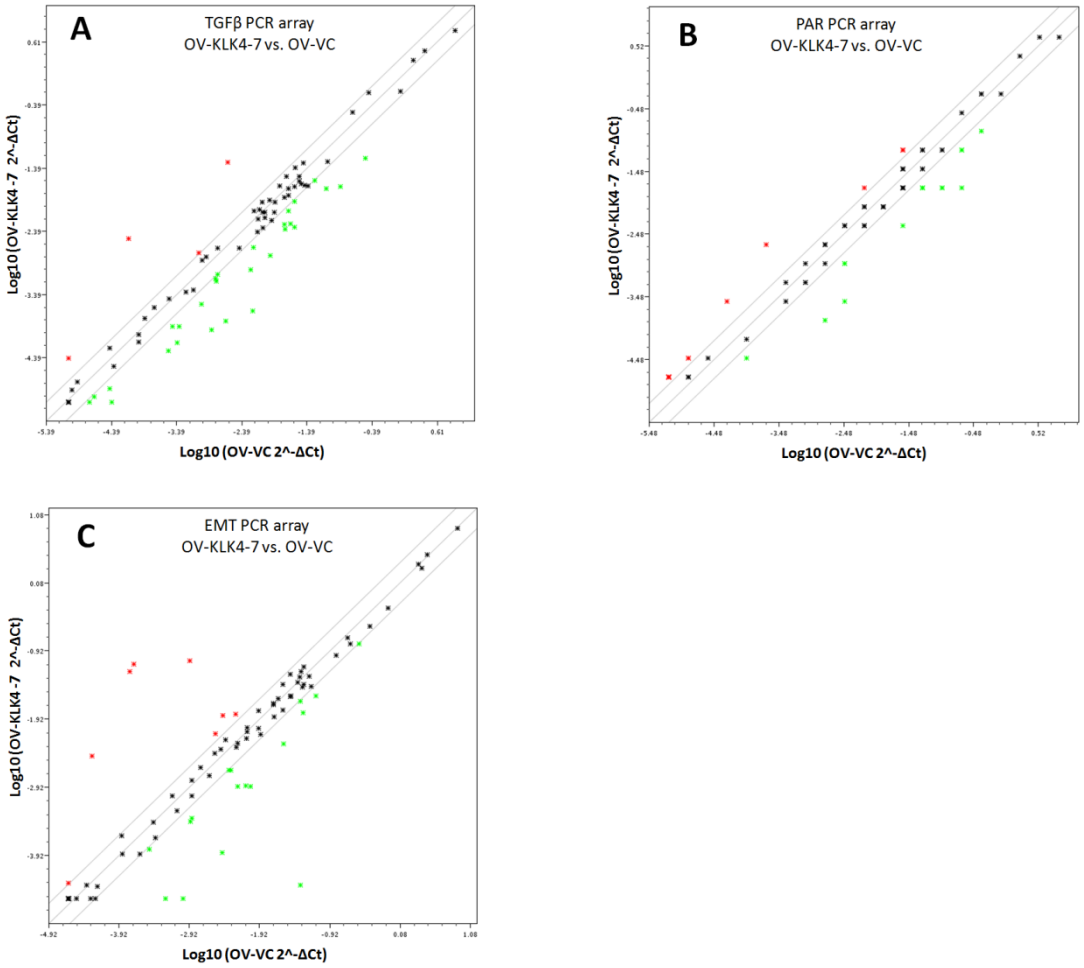

Figure 7. Gene expression as analyzed using the TGFβ (A), PAR (B) and EMT (C) PCR arrays in OV-KLK4-7 *vs.* OV-VC cells

Each dot represents at least one gene in the PCR arrays. Values on X and Y axes reflect the ΔCt values of each gene normalized to the house keeping genes in OV-VC and in OV-KLK4-7 cells, respectively (ΔCt values from bottom/left to top/right: about 18 to about -4). Genes with Ct values >35 were excluded. Black dots represent genes showing less than 2-fold regulation (less than a difference of 1.0 in ΔCt values) in OV-KLK4-7 *vs.* OV-VC cells. Red/green dots represent genes showing more than a 2-fold upregulation/downregulation in OV-KLK4-7 *vs.* OV-VC cells. The distance of a dot from the central diagonal is an indicator for the difference in gene regulation.

4.2.1 Screening of genes showing a more than 2-fold regulation in OV-KLK4-7 compared to OV-VC cells by the TGFβ PCR array

Using the TGFβ PCR array (**Table 7**), 32 out of 84 genes (38.1%) were found to be distinctly regulated (>2-fold difference). Among them, only 4 genes (12.5%) were upregulated, while 28 (87.5%) showed downregulation in OV-KLK4-7 *vs.* OV-VC cells. Fifteen genes were regulated >4-fold, 5 genes >10-fold. The gene displaying the

highest score was COL1A2 (41.64-fold upregulation). These data indicate that overexpression of KLK4-7 has distinct effects on TGFβ pathway genes in OV-MZ-6 cells.

The genes were further classified into 3 categories: OKAY, A and B according to the Ct values. Category OKAY means that the gene's threshold cycle is reasonably low (<30, which corresponds to a comparatively high gene expression) in both OV-VC and OV-KLK4-7 cells. Category A means that the gene's threshold cycle is relatively high (30-35, comparatively low gene expression) in either OV-VC or OV-KLK4-7 cells, and is low (<30) in the other cell line. Category B means that the gene's threshold cycle is relatively high (30-35) in both OV-VC and OV-KLK4-7 cells. Generally, PCR array results of genes categorized as OKAY and A are reliable, whereas genes with low expression in both cell lines are often misclassified as regulated genes due to the higher variability of the PCR results. In the TGFβ PCR array, 19 of the >2-fold regulated genes were labeled as OKAY, 8 as A and 5 as B (**Table 7**).

4.2.2 Screening of genes showing a more than 2-fold regulation in OV-KLK4-7 compared to OV-VC cells by the PAR PCR array

Applying the PAR PCR array (**Table 8**), 23 out of 84 genes (27.4%) were found to be distinctly regulated (>2-fold). Among them, 8 genes (34.8%) were upregulated and 15 (65.2%) showed downregulation. Eight genes displayed a more than 4-fold, 4 genes a more than 10-fold expression difference. Sixteen genes were grouped into category OKAY, 3 into category A, and 4 into category B. Overall, in the PAR PCR array the fold change values were relatively small compared with those of the TGFβ PCR array.

Table 7. Genes displaying a more than 2-fold regulation in OV-KLK4-7 compared to OV-VC cells in the TGFβ PCR array

Gene symbol	Gene description	Fold change	Category
BAMBI	BMP and activin membrane-bound inhibitor homolog	-4.23	OKAY
BMP5	Bone morphogenetic protein 5	20.25	OKAY
EMP1	Epithelial membrane protein 1	-4.26	OKAY
ID1	Inhibitor of DNA binding 1	-5.46	OKAY
ID2	Inhibitor of DNA binding 2	-5.50	OKAY
IL6	Interleukin 6 (interferon, beta 2)	-5.54	OKAY
TGFBI	Transforming growth factor, beta-induced, 68kDa	-6.63	OKAY
TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	-4.14	OKAY
JUNB	Jun B proto-oncogene	-6.36	OKAY
ACVRL1	Activin A receptor type II-like 1	2.11	OKAY
BMP6	Bone morphogenetic protein 6	-2.50	OKAY
HERPUD1	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	-2.19	OKAY
PLAU	Plasminogen activator, urokinase	-2.04	OKAY
SMAD3	SMAD family member 3	-2.17	OKAY
TGIF1	TGFB-induced factor homeobox 1	-2.46	OKAY
THBS1	Thrombospondin 1	-3.48	OKAY
KLHL24	Kelch-like 24 (Drosophila)	-2.69	OKAY
NOV	Nephroblastoma overexpressed gene	-3.41	OKAY
SMAD6	SMAD family member 6	-2.07	OKAY
BMP4	Bone morphogenetic protein 4	-12.21	A
CHRD	Chordin	-3.46	A
COL1A2	Collagen, type I, alpha 2	41.64	A
EGR2	Early growth response 2	-26.35	A
LTBP1	Latent transforming growth factor beta binding protein 1	-14.72	A
PDGFB	Platelet-derived growth factor beta polypeptide	-5.82	A
SMAD7	SMAD family member 7	-5.94	A
SOX4	SRY (sex determining region Y)-box 4	-2.73	A
BMP2	Bone morphogenetic protein 2	4.56	B
BMPER	BMP binding endothelial regulator	-5.17	B
GDF5	Growth differentiation factor 5	-2.93	B
GDF7	Growth differentiation factor 7	-2.27	B
KANK4	KN motif and ankyrin repeat domains 4	-2.31	B

OKAY: This gene's threshold cycle is <30 in both OV-VC and OV-KLK4-7 cells. **A**: This gene's threshold cycle is relatively high (30-35) in either OV-VC or OV-KLK4-7 cells, and is <30 in the other cell line. **B**: This gene's threshold cycle is relatively high (30-35) in both OV-VC and OV-KLK4-7 cells. Red: upregulated in OV-KLK4-7 *vs.* OV-VC cells; blue: downregulated in OV-KLK4-7 *vs.* OV-VC cells.

Table 8. Genes showing a more than 2-fold regulation in OV-KLK4-7 compared to OV-VC cells in the PAR PCR array

Symbol	Description	Fold change	Category
EGR1	Early growth response 1	-6.06	OKAY
ICAM1	Intercellular adhesion molecule 1	-6.06	OKAY
JUNB	Jun B proto-oncogene	-12.13	OKAY
THBS1	Thrombospondin 1	-6.06	OKAY
CTGF	Connective tissue growth factor	-3.03	OKAY
CYR61	Cysteine-rich, angiogenic inducer, 61	-3.03	OKAY
F2R	Coagulation factor II (thrombin) receptor	-3.03	OKAY
HBEGF	Heparin-binding EGF-like growth factor	2.64	OKAY
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	2.64	OKAY
IL6	Interleukin 6 (interferon, beta 2)	-3.03	OKAY
ITGB1	Integrin, beta 1	-3.03	OKAY
PLAU	Plasminogen activator, urokinase	-3.03	OKAY
PTPN11	Protein tyrosine phosphatase, non-receptor type 11	2.64	OKAY
SERPINB5	Maspin	-3.03	OKAY
TNF	Tumor necrosis factor	-3.03	OKAY
TP53	Tumor protein p53	2.64	OKAY
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	-12.13	A
F10	Coagulation factor X	10.56	A
MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	-12.13	A
F2RL2	Coagulation factor II (thrombin) receptor-like 2	5.28	B
IL13	Interleukin 13	2.64	B
ITGAM	Integrin, alpha M	-3.03	B
SERPINB2	Plasminogen activator inhibitor-2 (PAI-2)	2.64	B

OKAY: This gene's threshold cycle is reasonably low in both OV-VC and OV-KLK4-7 (<30). **A**: This gene's threshold cycle is relatively high (30-35) in either OV-VC or OV-KLK4-7, and is reasonably low in the other cell line (<30). **B**: This gene's threshold cycle is relatively high (30-35) in both OV-VC and OV-KLK4-7. Red: upregulated in OV-KLK4-7 *vs.* OV-VC cells; blue: downregulated in OV-KLK4-7 *vs.* OV-VC cells.

4.2.3 Screening of genes showing a more than 2-fold regulation in OV-KLK4-7 compared to OV-VC cells by the EMT PCR array

Using the EMT PCR array (**Table 9**), 25 out of 84 genes (29.8%) were found distinctly regulated (›2-fold). Among them, 8 genes (32.0%) were upregulated and 17 genes (68.0%) showed downregulation. In contrast to the TGFβ array, the percentage

of upregulated genes in the EMT array was clearly higher (32.0% *vs.* 12.5%). Twelve genes displayed a more than 4-fold, 8 genes a more than 10-fold expression regulation within the EMT PCR array. The gene displaying the highest score was KRT7 (-1,038.29), which can be attributed to the fact that KRT7 is not expressed, or only in minute amounts, in OV-KLK4-7 cells. Fourteen genes were grouped into category OKAY, 8 into category A, and 3 into category B. Overall, the observed fold change values were the most pronounced within the three different applied PCR arrays. It seems that overexpression of KLK4-7 has very strong modulating effects on EMT genes in OV-MZ-6 cells.

4.3 Selection of regulated candidate genes in KLK4-7-expressing *vs.* vector control OV-MZ-6 ovarian cancer cells

Based on the results of the 3 PCR array analyses, a total of 74 genes were found to be more than 2-fold regulated in OV-KLK4-7 *vs.* OV-VC cells (**Table 10**). In addition, our collaborators from the Queensland University of Technology (QUT) in Brisbane, Australia, analyzed the global mRNA expression pattern in OV-KLK4-7 *vs.* OV-VC cells by the Agilent Human Genome CGH Microarray technique (D. Loessner, unpublished data). The *Top 10* upregulated and downregulated genes, identified by this screen, are shown in the **Appendix 10.4**. Furthermore, another collaboratoring group from the University of Freiburg, Germany, compared the proteome profiles of cell conditioned medium of OV-KLK4-7 cells with that of OV-VC cells using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [173]. In this screen, 45 proteins, annotated as secreted soluble or membrane-associated proteins, were identified (**Appendix 10.5**). However, in these analyses also further proteins, *e.g.* cytosolic proteins released by dead, disrupted cell (due to growth of the cell lines in FCS-free medium) were found, the relative abundance of which in the two cell lines may point to a differential regulation (O. Schilling, personal communication). We, therefore, considered the independent genome-wide RNA microarray and proteomics data to select 10 candidate genes for further analyses (**Table 10, 11**).

Table 9. Genes showing a more than 2-fold regulation in OV-KLK4-7 compared with OV-VC cells in the EMT PCR array

Symbol	Description	Fold change	Comment
CAMK2N1	Endogenous inhibitor of CAMKII	-7.26	OKAY
COL5A2	Collagen, type V, alpha 2	71.01	OKAY
FN1	Fibronectin 1	-6.02	OKAY
RGS2	Regulator of G-protein signaling 2, 24kDa	-5.06	OKAY
TSPAN13	Tetraspanin 13	-4.72	OKAY
AHNAK	AHNAK nucleoprotein	3.81	OKAY
DSP	Desmoplakin	2.58	OKAY
FZD7	Frizzled family receptor 7	-2.06	OKAY
IGFBP4	Insulin-like growth factor binding protein 4	-2.93	OKAY
ITGAV	Integrin, alpha V	-3.36	OKAY
ITGB1	Integrin, beta 1	-2.03	OKAY
JAG1	Jagged 1	-2.19	OKAY
PLEK2	Pleckstrin 2	2.58	OKAY
TIMP1	TIMP metallopeptidase inhibitor 1	-2.08	OKAY
COL1A2	Collagen, type I, alpha 2	69.55	A
DSC2	Desmocollin 2	-3.32	A
KRT19	Keratin 19	347.29	A
KRT7	Keratin 7	-1038.29	A
MSN	Moesin	396.18	A
SNAI1	Snail homolog 1	-3.12	A
STEAP1	Six transmembrane epithelial antigen of the prostate 1	-35.26	A
WNT5B	Wnt-5b	-26.72	A
MMP3	Matrix metallopeptidase 3 (stromelysin 1)	2.07	B
MMP9	Matrix metallopeptidase 9	-2.17	B
ZEB2	Zinc finger E-box binding homeobox 2	-19.70	B

OKAY: This gene's threshold cycle is reasonably low in both OV-VC and OV-KLK4-7 (<30). **A**: This gene's threshold cycle is relatively high (30-35) in either OV-VC or OV-KLK4-7, and is reasonably low in the other cell line (<30). **B**: This gene's threshold cycle is relatively high (30-35) in both OV-VC and OV-KLK4-7. Red: upregulated in OV-KLK4-7 *vs.* OV-VC cells; blue: downregulated in OV-KLK4-7 *vs.* OV-VC cells.

Table 10. Full list of genes showing a more than 2-fold regulation in OV-KLK4-7 compared with OV-VC cells

PCR array data		Genome-wide RNA microarray data (regulation)	Proteomics data (fold change)
Gene symbol	Fold change		
ACVRL1	2.11	up	
AHNAK	3.81	no	1.52
BAMBI	-4.23	no	
BMP2	4.56	no	
BMP4	-12.21	down	
BMP5	20.25	up	
BMP6	-2.50	down	
BMPER	-5.17	no	
CAMK2N1	-7.26	no	
CHRD	-3.46	no	
COL1A2*	41.64	up	
COL5A2	71.01	up	30.48
CSF2	-12.13	no	
CTGF	-3.03	no	-1.61
CYR61	-3.03	down	
DSC2	-3.32	down	
DSP	2.58	down	
EGR1	-6.06	no	
EGR2	-26.35	no	
EMP1	-4.26	down	
F10	10.56	up	
F2R	-3.03	no	
F2RL2	5.28	no	
FN1	-6.02	no	-1.64
FZD7	-2.06	no	
GDF5	-2.93	no	
GDF7	-2.27	no	
HBEGF	2.64	no	
HERPUD1	-2.19	no	
HMGCR	2.64	up	
ICAM1	-6.06	no	
ID1	-5.46	no	
ID2	-5.50	no	
IGFBP4	-2.93	no	
IL13	2.64	no	
IL6*	-5.54	no	
ITGAM	-3.03	no	

ITGAV	-3.36	down	
ITGB1*	-2.03	down	-1.35
JAG1	-2.19	no	
JUNB*	-6.36	down	
KANK4	-2.31	no	
KLHL24	-2.69	no	
KRT19	347.29	up	8.82
KRT7	-1038.29	down	
LTBP1	-14.72	down	
MMP1	-12.13	no	-1.37
MMP3	2.07	no	
MMP9	-2.17	no	
MSN	396.18	up	7.11
NOV	-3.41	no	
PDGFB	-5.82	no	
PLAU*	-2.04	no	1.01
PLEK2	2.58	up	
PTPN11	2.64	no	
RGS2	-5.06	no	
SERPINB2	2.64	no	
SERPINB5	-3.03	no	-1.02
SMAD3	-2.17	no	
SMAD6	-2.07	no	
SMAD7	-5.94	no	
SNAI1	-3.12	no	
SOX4	-2.73	no	
STEAP1	-35.26	down	
TGFBI	-6.63	no	-2.33
TGFBR2	-4.14	down	
TGIF1	-2.46	no	
THBS1*	-3.48	down	
TIMP1	-2.08	no	2.38
TNF	-3.03	up	
TP53	2.64	no	
TSPAN13	-4.72	down	
WNT5B	-26.72	down	
ZEB2	-19.70	down	

* These genes were located on more than one of the used PCR arrays. In all of these cases, regulation of gene expression was found on both PCR arrays: COL1A2, 41.64 (TGFβ array) *vs.* 69.55 (EMT array); IL6, -5.54 (TGFβ) *vs.* -3.03 (PAR); ITGB1, -2.03 (EMT) *vs.* -3.03 (PAR); JUNB, -6.36 (TGFβ) *vs.* -12.13 (PAR); PLAU, -2.04 (TGFβ) *vs.* -3.03 (EMT) and THBS1, -3.48 (TGFβ) *vs.* -6.06 (EMT).

Differential regulation of COL5A2, KRT19 and MSN was identified in all of the 3 independent analyses (PCR array, genome wide mRNA analysis and proteomics) and the other candidate genes were identified by two of the three independent analyses. Candidate genes from the EMT PCR array (MSN, KRT7, KRT19, COL1A2 and COL5A2) showed the most distinct expression difference. Genes from the PAR PCR array showed the lowest expression difference in OV-KLK4-7 compared with OV-VC cells. COL1A2 and JUNB were identified as regulated genes in two different PCR arrays (TGFβ plus EMT and TGFβ plus PAR, respectively) and the observed fold change values were similar. In addition, the bone morphogenetic proteins BMP4/BMP5 (down *vs.* up) and the proteases MMP1/F10 (down *vs.* up) showed an inverse expression pattern when compared to each other. These genes, listed in **Table 11**, were selected for further analysis.

Table 11. Candidate genes regulated in OV-KLK4-7 *vs.* OV-VC cells for further analysis

Gene symbol	Protein	Regulation	X-fold	PCR array
BMP4	Bone morphogenetic protein 4	**down**	**-12**	TGFβ
BMP5	Bone morphogenetic protein 5	**up**	**20**	TGFβ
*COL1A2	Collagen alpha-2(I) chain	**up**	**42**	TGFβ, EMT
COL5A2	Collagen alpha-2(V) chain	**up**	**71**	EMT
KRT7	Cytokeratin-7	**down**	**-1,038**	EMT
KRT19	Cytokeratin-19	**up**	**347**	EMT
MSN	Moesin	**up**	**396**	EMT
*JUNB	Transcription factor jun-B	**down**	**-12**	TGFβ, PAR
MMP1	Matrix metalloproteinase-1	**down**	**-12**	PAR
F10	Coagulation Factor X	**up**	**11**	PAR

* These genes were located on two of the used PCR arrays: COL1A2, 42 (TGFβ array) and 70 (EMT array); JUNB, -12 (PAR) and -6 (TGFβ).

4.4 Validation of potentially differentially regulated genes by qPCR analysis

The PCR array analyses were performed to identify candidate genes being differentially regulated in OV-KLK4-7 *vs.* OV-VC cells. For validation of the PCR array results (which are derived from single determinations), qPCR analysis using different probes and independently prepared RNA and cDNA of the cells was performed. The following genes showed upregulation in OV-KLK4-7 compared with OV-VC by both qPCR and PCR array analyses: BMP5 (10.5 ± 2.0 [qPCR] *vs.* 20.3 [PCR array], **Figure 8A**); COL1A2 (42.1 ± 14.8 *vs.* 69.6, **Figure 8B**); COL5A2 (38.6 ± 11.3 *vs.* 71.0, **Figure 8C**); KRT19 (256.5 ± 56.5 *vs.* 347.3, **Figure 8D**); MSN (313.9 ± 59.0 *vs.* 396.2, **Figure 8E**) and F10 (8.3 ± 2.3 *vs.* 10.6, **Figure 8F**). The following genes showed downregulation in OV-KLK4-7 compared with OV-VC cells by both qPCR and PCR array analyses: BMP4 (0.18 ± 0.02 *vs.* 0.08, **Figure 9A**); KRT7 (0.0020 ± 0.0008 *vs.* 0.0010, **Figure 9B**); JUNB (0.15 ± 0.07 *vs.* 0.08, **Figure 9C**) and MMP1 (0.15 ± 0.03 *vs.* 0.08, **Figure 9D**). Thus, the qPCR results were in accordance with the PCR array results and, furthermore, the observed fold change values were similar. Therefore, on the mRNA level, all of the 10 candidate genes were found to be significantly diffentially regulated in OV-KLK4-7 *vs.* OV-VC cells.

4.5 Establishment of a three-dimensional (3D) OV-MZ-6 cell culture model

The PCR array and qPCR results demonstrated that overexpression of KLK4-7 modulates expression of other cancer-related genes in OV-MZ-6 cells. These findings were based on analyses using RNA extracted from monolayer cells. We were interested to evaluate whether the gene regulation effects can also be observed when the cancer cells are grown in three-dimensional (3D) spheroids. 3D cell cultures of tumor cells may represent a cellular micro-tumor model that more closely mimics the function of the tumor environment *in vivo* [174]. Therefore, we established a 3D OV-MZ-6 cell culture model based on the use of the novel semi-synthetic biomaterial, gelatin methacrylamide-based (GelMA) hydrogel [174]. OV-VC and OV-KLK4-7 cells were cultured at a concentration of 3 x 10^5 cells per ml GelMA hydrogel for 2 weeks as described in the Materials and Methods section. At day 1, the (mostly singular) cells were about 10 μm in diameter. As shown in **Figure 10**, two weeks after seeding, multicellular spheroids of OV-VC and OV-KLK4-7 cells had formed,

reaching a diameter of 60 µm and 80 µm, respectively.

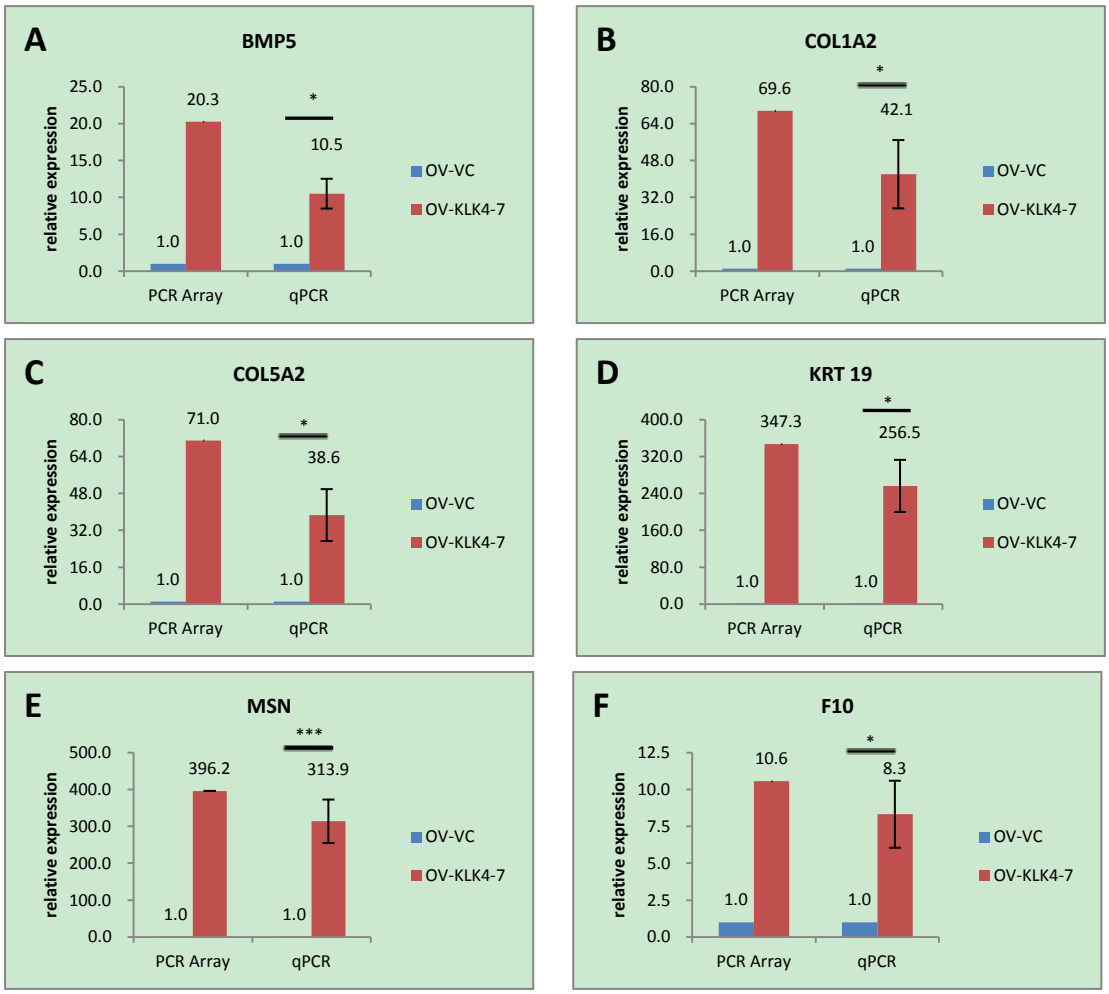

Figure 8. Analysis of mRNA expression in OV-KLK4-7 *vs.* OV-VC cells using PCR array and qPCR analysis, respectively

Candidate genes identified upregulation in OV-KLK4-7 *vs.* OV-VC cells by PCR array analyses were validated by qPCR analysis using independently isolated RNA from the cells. OV-KLK4-7 cells displayed a significant upregulation of gene expression of BMP5 (A), COL1A2 (B), COL5A2 (C), KRT19 (D), MSN (E) and F10 (F) compared to OV-VC cells. *, $p \leq 0.05$; ***, $p \leq 0.001$.

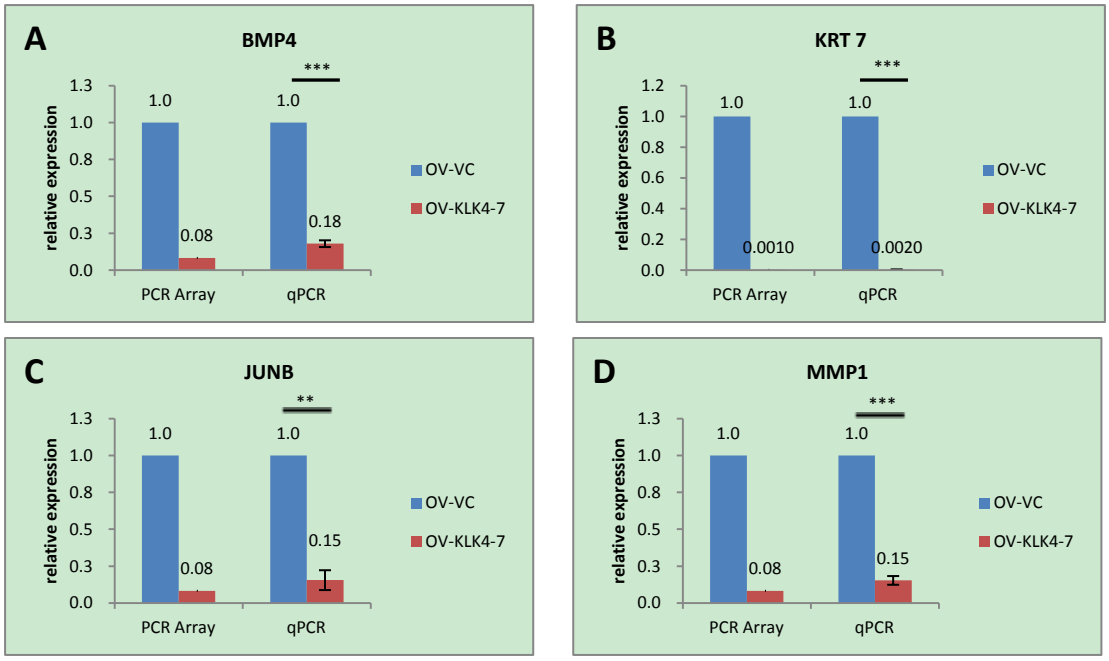

Figure 9. Analysis of mRNA expression in OV-KLK4-7 *vs.* OV-VC cells using PCR array and qPCR analysis, respectively

Candidate genes identified downregulation in OV-KLK4-7 *vs.* OV-VC cells by PCR array analyses were validated by qPCR analysis using independently isolated RNA from the cells. OV-KLK4-7 cells displayed a significant downregulation of the genes BMP4 (A), KRT7 (B), JUNB (C) and MMP1 (D) compared to OV-VC cells. **, $p \leq 0.01$; ***, $p \leq 0.001$.

After 2 weeks, hydrogels containing cell spheroids were collected and RNA was extracted from the gels (10 hydrogel pieces per RNA sample). **Table 12** depicts the yields of RNA extracted from 3D cell spheroids and from cells cultured in 2D monolayer. Both the quantity and quality of 3D-derived RNA was not as good as that of 2D-derived RNA. This could be due to the rather low number of cells embedded in the proteineous matrix in the 3D system.

Another problem was that cells in hydrogels not only formed spheroids, but also grew on the surfaces of gels as monolayers, especially at the bottom of the hydrogels. Therefore, the extracted RNA from the 3D-model is not only derived from spheroids but also from cells growing as 2D monolayers (cells on the surfaces of gels). Although this novel 3D cell culture model may be promising, *e.g.* for analysis of protein expression in spheroids by immunohistochemical techniques (which allows localization of the analyzed proteins in spheroids only), it seems to be not well suited for mRNA quantification by qPCR. Therefore, further analyses of the expression

pattern of the candidate genes focused on validation studies on the protein level.

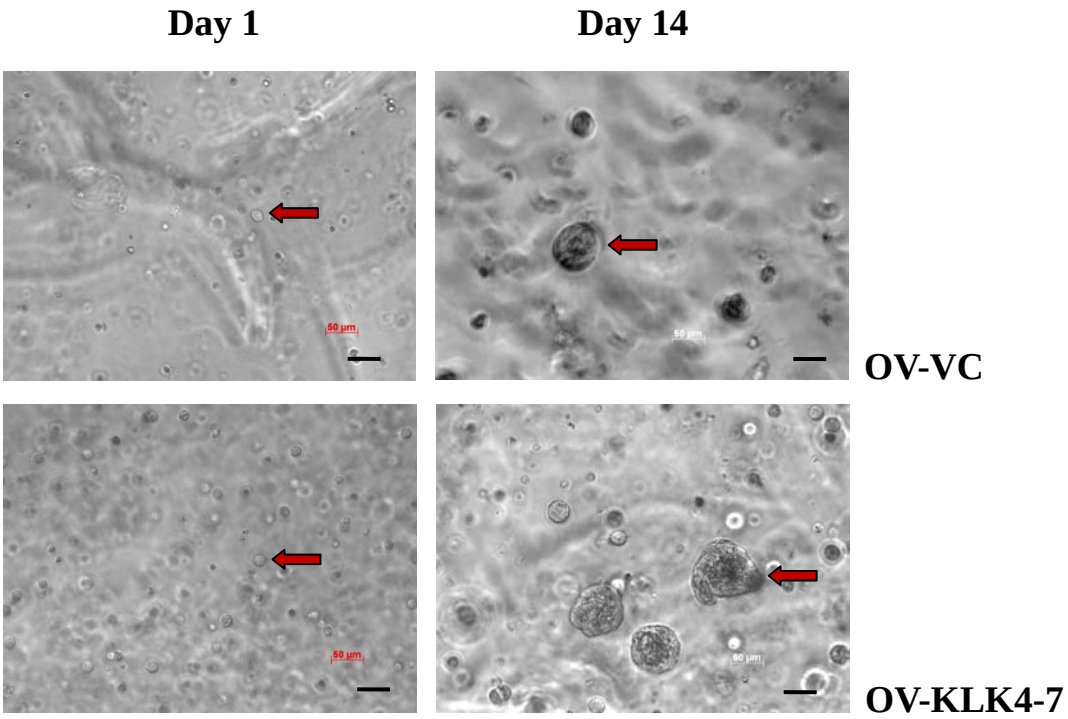

Figure 10. 3D culture of OV-VC and OV-KLK4-7 cells
OV-VC and OV-KLK4-7 cells were cultured in GelMA-based hydrogels at a concentration of $3x10^5$ cells/ml. At day 1, the cells were singular, with a diameter of about 10 μm (indicated by arrows). Two weeks after seeding, the multicellular spheroids of OV-VC and OV-KLK4-7 cells had reached a diameter of 60 μm and 80 μm, respectively (indicated by arrows). Bar: 50 μm.

Table 12. Yields of RNA after extraction from OV-MZ-6 cells cultured in 3D and 2D models, respectively

RNA Sample	Concentration (ng/ul)	A260/280	A260/230
3D			
OV-VC	12.83	1.59	0.56
OV-KLK4-7	8.82	1.46	0.37
2D			
OV-VC	310.56	2.10	2.04
OV-KLK4-7	547.62	2.08	2.15

An A260/280 ratio of about 2.0 indicates a pure RNA preparation. If the ratio is distinctly lower, it may indicate the presence of contaminants such as proteins that absorb at or near 280 nm. Similarly, an A260/230 ratio in the range of 2.0 – 2.2 is expected for appropriate RNA preparations.

4.6 Validation of differential expression of MSN, KRT7, KRT19 and JUNB in OV-KLK4-7 *vs.* OV-VC cells by Western blot analysis

In addition to the validation of the PCR array results by qPCR, four genes (MSN, KRT7, KRT19 and JUNB) were selected for further analysis by Western blotting. Protein was extracted from cell lysates as described in the **Materials and Methods** section and Western blot analysis was performed to investigate protein expression of the four candidates in OV-KLK4-7 *vs.* OV-VC cells. **Figure 11A** shows a Coomassie blue-stained protein gel with OV-VC and OV-KLK4-7 protein samples as loading control for Western blot analysis. On the protein level, a strong upregulation of MSN (**Figure 11B**) and KRT 19 (**Figure 11C**), together with downregulation of JUNB (**Figure 11D**) and KRT7 (**Figure 11E**) was observed in OV-KLK4-7 *vs.* OV-VC cells. Downregulation of JUNB expression seemed to be more pronounced on the protein than on mRNA level in OV-KLK4-7 cells, which could be due to a post-transcriptional regulation event (**Figure 11D, Figure 9C**).

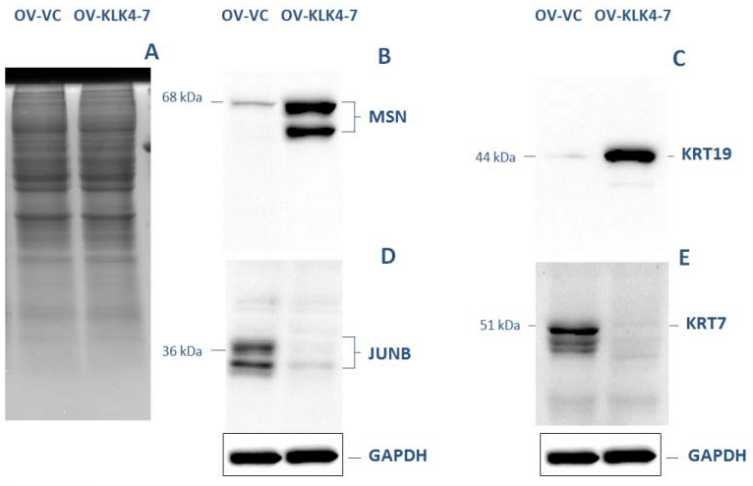

Figure 11. Differential protein expression of MSN, KRT7, KRT19, and JUNB in OV-KLK4-7 *vs.* OV-VC cells as analyzed by Western blotting

A: Coomassie blue staining showed no obvious difference between OV-VC and OV-KLK4-7 protein samples (40 µg/lane). MSN (B) and KRT 19 (C) are strongly upregulated in OV-KLK4-7 compared to OV-VC cells. JUNB (D) and KRT 7 (E) are strongly downregulated in OV-KLK4-7 compared to OV-VC cells. Additional bands with a lower apparent molecular weight in (B), (D) and (E) are probably representing degradation products of the respective proteins. GAPDH was used as reference.

4.7 Validation of differential expression of MSN, KRT7, KRT19 and JUNB in OV-KLK4-7 *vs.* OV-VC cells by immunofluorescence

In addition to the Western blot analysis, protein expression of the 4 genes (MSN, KRT19, KRT7 and JUNB) was examined by immunofluorescence. OV-VC and OV-KLK4-7 cells were stained with the antibodies directed to one of the four proteins and a stronger staining of MSN and KRT19, together with weaker staining of KRT7 and JUNB in OV-KLK4-7 *vs.* OV-VC cells was clearly observed (**Figure 12**). The staining pattern of the 4 proteins was quite different. Moesin showed a clear membrane staining in OV-MZ-6 cells while JUNB was mainly located inside the nuclei in OV-VC cells. This was expected as moesin is a membrane-organizing extension spike protein whereas JUNB is a transcriptional factor. Even though the two keratins are cytoplasmic proteins, differences in the staining patterns were observed. KRT19 showed a staining pattern throughout the cytoplasm whereas KRT7 was concentrated as dots associated with the cell nuclei. In summary, these results further confirmed the previous findings concerning differential gene/protein expression of these four factors in OV-KLK4-7 *vs.* OV-VC cells.

4.8 Validation of differential expression of MSN, KRT7, KRT19 and JUNB in OV-KLK4-7- *vs.* OV-VC-derived tumor tissue by immunohistochemistry

In a previous study, OV-VC and OV-KLK4-7 cells had been injected into nude mice to form tumors *in vivo* [172]. Formalin-fixed, paraffin-embedded tumor tissue from this study was now used for immunohistochemical analysis of protein expression of MSN, KRT19, KRT7 and JUNB. Again, a much stronger staining of MSN and KRT19, together with a weaker staining of KRT7 and JUNB was observed in OV-KLK4-7 cells compared to OV-VC cells (**Figure 13**). Moreover, these proteins showed different staining patterns in the tumor tissues as well. KRT19 and moesin staining was rather homogeneous within the tissues while KRT7 showed a spot-staining pattern. JUNB was clearly located inside the nuclei of tumor cells. Concerning the expression patterns in OV-KLK4-7 *vs.* OV-VC cells, these results further confirmed the findings from the PCR array, qPCR, Western blot and immunofluorescence analyses.

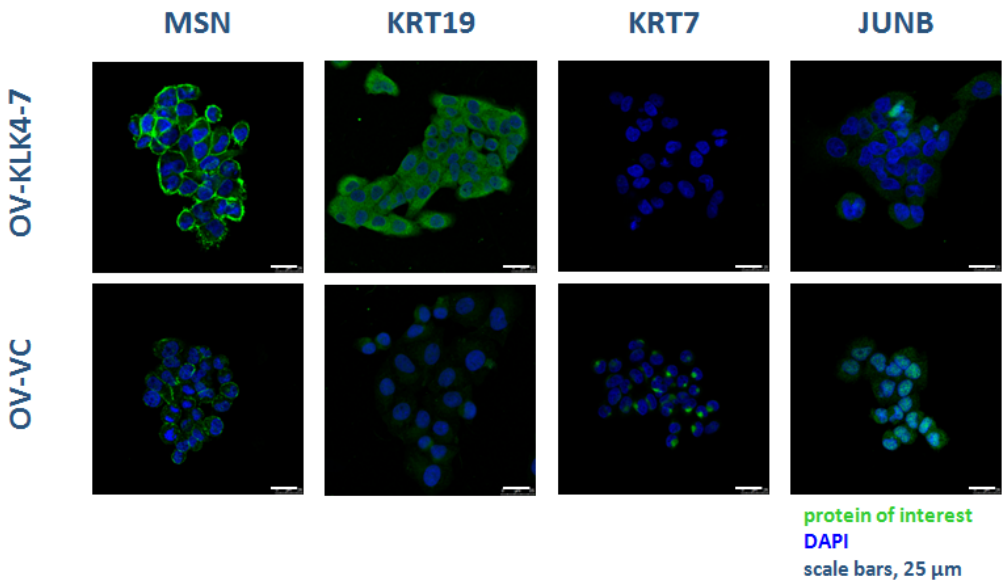

Figure 12. Differential protein expression of MSN, KRT7, KRT19, and JUNB in OV-KLK4-7 *vs.* OV-VC cells as analyzed by immunofluorescence
OV-VC and OV-KLK4-7 cells were stained with antibodies directed to either MSN, KRT19, KRT7 or JUNB. DAPI was used to stain the cell nuclei. A stronger staining of MSN and KRT19, together with weaker staining of KRT7 and JUNB in OV-KLK4-7 *vs.* OV-VC cells was observed. Moesin showed clear membrane staining in cells while JUNB was mainly located inside the nuclei in OV-VC cells. KRT19 showed a cytoplasm-based staining pattern whereas KRT7 was visible as dots associated with the cell nuclei.

4.9 Characterization of moesin (MSN) protein expression in human tissues

Since moesin (MSN) was found to be strongly upregulated on both the mRNA and protein level in OV-KLK4-7 *vs.* OV-VC cells, we were interested to establish an immunohistochemical protocol for human tissue to follow expression of moesin in normal tissue as well as tumor tissue of ovarian cancer patients. For this, we used the anti-moesin-antibody HPA011135 and tested its usefulness on tonsil tissue as positive control and colon tissue as negative control (see Human Protein Atlas, MSN, Antibody HPA011135). As expected, a strong moesin staining was observed in tonsil tissue, but moesin was not detectable in colon glandular cells (**Figure 14**).

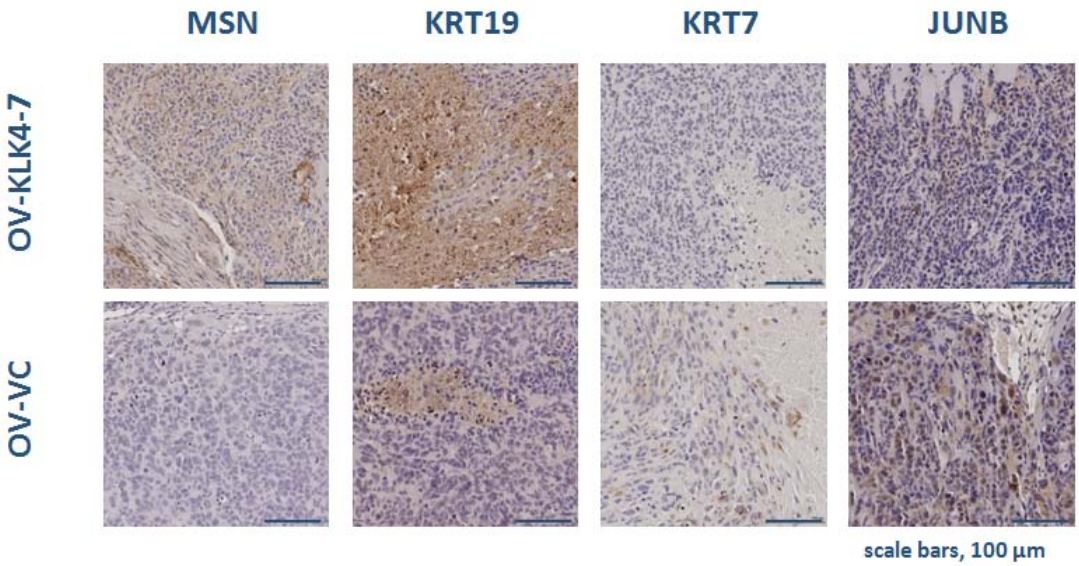

Figure 13. Differential protein expression of MSN, KRT7, KRT19, and JUNB in OV-KLK4-7-derived *vs.* OV-VC-derived tumor tissue as analyzed by immuno-histochemistry

OV-VC and OV-KLK4-7 cells had previously been injected into the peritoneum of nude mice to form tumors *in vivo* [172]. The formalin-fixed, paraffin-embedded tumor tissue was used for immunohistochemical analysis of protein expression of MSN, KRT19, KRT7 and JUNB. In tumor tissue derived from OV-KLK4-7 cells, a much stronger staining of MSN and KRT19, together with a weaker staining of KRT7 and JUNB was observed when compared with the staining pattern of OV-VC-derived tumors.

Next, we stained ovarian cancer samples. Moesin was reported to be absent in normal ovary tissue (Human Protein Atlas, MSN, Antibody HPA011135). The staining patterns of the ovarian cancer tissue samples indicated high and moderate moesin expression, respectively. Thus, these results may suggest that moesin expression is increased in ovarian cancer tissues as compared with normal ovary.

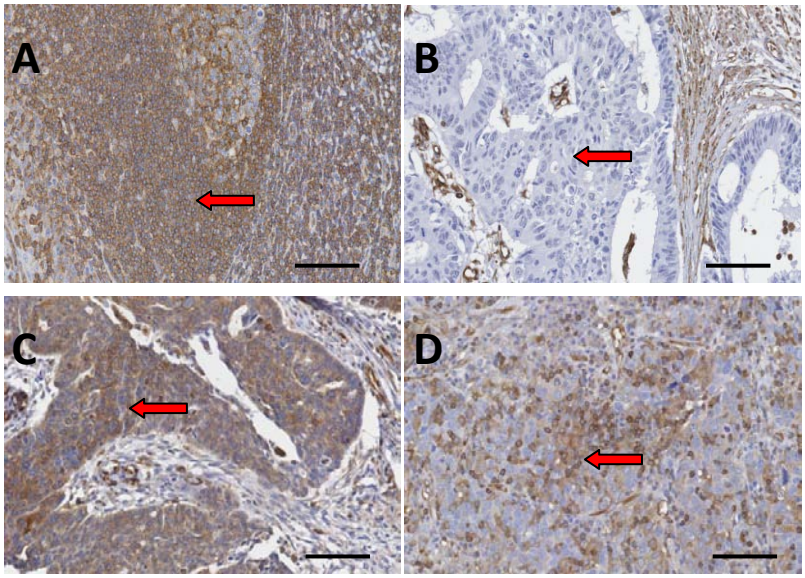

Figure 14. Detection of moesin (MSN) in various human tissues by immunohistochemistry

The formalin-fixed, paraffin-embedded tissue was used for immunohistochemical analysis of protein expression of moesin. Moesin was strongly stained in normal tonsil cells **(A,** indicated by the arrow**)** and not detectable in normal colon glandular cells **(B,** indicated by the arrow**)**. Ovarian cancer cells (pT3c, **(C)**; pT1a **(D),** indicated by arrows) showed high and moderate moesin staining, respectively. Bar 100 µm.

4.10 Comparison of the migratory characteristics of OV-KLK4-7 and OV-VC cells

The above described results demonstrate that overexpression of KLK4-7 in OV-MZ-6 ovarian cancer cells modulates expression of other tumor-relevant genes, among them MSN, KRT7, KRT19 and JUNB. These genes are associated with cell structure and cell integrity. Therefore, we were interested to analyze whether KLK4-7 expression affects the cell behavior with respect to cell migration. To analyze this, wound scratch assays were performed. Here, cell migration was documented by photos taken 0, 12, 24, and 48 h after introducing the scratches (**Figure 15**). Even after 48 h, the gap was not filled with cells. Futhermore, the two cell lines did not show obvious differences with respect to their migration ability.

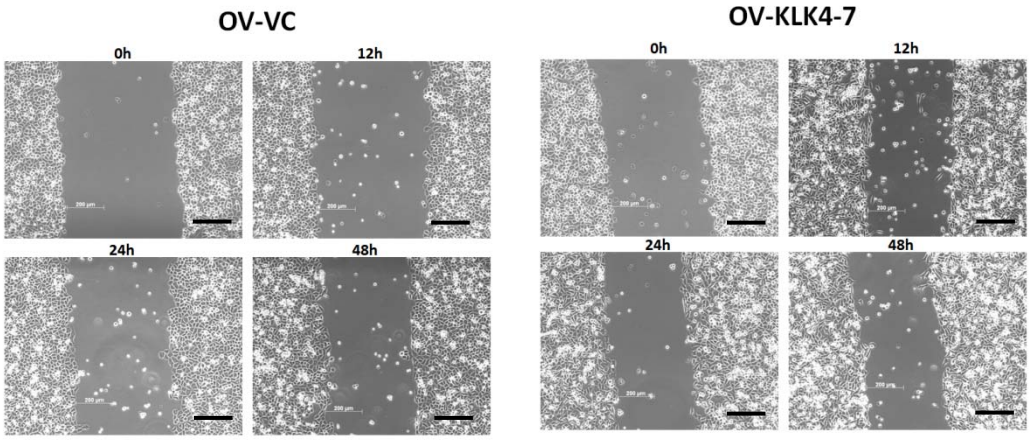

Figure 15. Migration assay with OV-VC and OV-KLK4-7 cells
Gaps were created with p200 pipet tips after the cells had formed a confluent monolayer, *i.e.* 4 d after seeding. Photos of the scratches were taken at 0, 12, 24, and 48 h after introducing the scratches. Even after 48 h, the gaps were not filled with cells. OV-VC and OV-KLK4-7 cells did not show obvious differences as to migration ability. Bar 200 μm.

4.11 Impact of KLK4 on gene regulation in OV-MZ-6 ovarian cancer cells

Previously, KLK4 has been demonstrated to regulate EMT in prostate cancer cells. Furthermore, KLK4 was also shown to be involved in PAR signaling in these cell types. Therefore, we hypothesized that KLK4 may largely be responsible for the effects on gene regulation seen in OV-KLK4-7 cells. Thus, we also performed the 3 PCR array (TGFβ, PAR and EMT) analyses using RNA derived from OV-KLK4 cells.

Using the TGFβ PCR array (**Table 13**), 17 out of 84 genes (19.0%) were found to be distinctly regulated in OV-KLK4 compared to OV-VC cells (>2-fold). Among them, 4 genes (25.0%) were upregulated and 12 genes (75.0%) showed downregulation in OV-KLK4 cells. Four genes showed a difference in gene expression of more than 4-fold, with BMP5 displaying the highest score (6.52). Seven genes were categorized as OKAY and 3 were attributed to category A. Six genes were labeled as category B, indicating low mRNA expression in OV-KLK4 and OV-VC cells.

Table 13. Genes showing more than a 2-fold regulation in OV-KLK4 *vs.* OV-VC cells in the TGFβ PCR array

Symbol	Description	Fold change	Comment
BMP5	Bone morphogenetic protein 5	6.52	OKAY
ID1	Inhibitor of DNA binding 1	-5.80	OKAY
ID2	Inhibitor of DNA binding 2	-4.74	OKAY
IGFBP3	Insulin-like growth factor binding protein 3	-2.58	OKAY
IL6	Interleukin 6 (interferon, beta 2)	-2.09	OKAY
NOG	Noggin	-2.94	OKAY
EGR2	Early growth response 2	-2.20	OKAY
JUNB	Jun B proto-oncogene	-2.78	OKAY
CHRD	Chordin	-3.80	A
FGF2	Fibroblast growth factor 2 (basic)	-3.57	A
SMAD3	SMAD family member 3	-2.06	A
AMH	Anti-Mullerian hormone	2.01	B
COL1A2	Collagen, type I, alpha 2	-2.52	B
DCN	Decorin	2.96	B
IGF1	Insulin-like growth factor 1 (somatomedin C)	2.45	B
LEFTY1	Left-right determination factor 1	2.06	B
TGFB2	Transforming growth factor, beta 2	-4.52	B

OKAY: This gene's threshold cycle is reasonably low in both OV-VC and OV-KLK4 (<30). A: This gene's threshold cycle is relatively high (30-35) in either OV-VC or OV-KLK4, and is reasonably low in the other sample (<30). B: This gene's threshold cycle is relatively high (30-35) in both OV-VC and OV-KLK4.

Applying the PAR PCR array (**Table 14**), 10 out of 84 genes (11.9%) were found distinctly regulated (>2-fold). Among them, 2 genes (20.0%) were upregulated, while 8 genes (80.0%) showed downregulation. Only one gene (MMP1) showed a >4-fold differential gene expression (-6.96). Six genes were labeled as OKAY, 3 as A and 1 as B.

Table 14. Genes showing more than a 2-fold regulation in OV-KLK4 *vs.* OV-VC cells in the PAR PCR array

Symbol	Description	Fold change	Comment
CXCL1	Chemokine (C-X-C motif) ligand 1	-3.48	OKAY
IL8	Interleukin 8	-3.48	OKAY
JUNB	Jun B proto-oncogene	-3.48	OKAY
SERPINB5	Maspin	-3.48	OKAY
TNF	Tumor necrosis factor	-3.48	OKAY
TP53	Tumor protein p53	2.30	OKAY
IL1B	Interleukin 1, beta	-3.48	A
MMP1	Matrix metallopeptidase 1	-6.96	A
NFATC1	Nuclear factor of activated T-cells	-3.48	A
F10	Coagulation factor X	2.30	B

OKAY: This gene's threshold cycle is reasonably low in both OV-VC and OV-KLK4 (<30). A: This gene's threshold cycle is relatively high (30-35) in either OV-VC or OV-KLK4, and is reasonably low in the other sample (<30). B: This gene's threshold cycle is relatively high (30-35) in both OV-VC and OV-KLK4.

Using the EMT PCR array (**Table 15**), only 9 out of 84 genes (10.7%) were found distinctly regulated (>2-fold). Among them, 3 genes (33.3%) were upregulated and 6 genes (66.7%) showed downregulation. Only one gene (TGFB2) showed a difference of more than 4 fold (-4.92). Five genes were labeled as OKAY, 1 as A and 3 as B.

Table 15. Genes showing more than a 2-fold regulation in OV-KLK4 *vs.* OV-VC cells in the EMT PCR array

Symbol	Description	Fold change	Comment
AHNAK	AHNAK nucleoprotein	3.92	OKAY
CAMK2N1	Inhibitor of CAMKII	-3.86	OKAY
DSP	Desmoplakin	2.71	OKAY
KRT14	Keratin 14	-2.03	OKAY
KRT7	Keratin 7	2.04	OKAY
COL5A2	Collagen, type V, alpha 2	-2.69	A
MMP9	Matrix metallopeptidase 9	-3.68	B
MSN	Moesin	-2.89	B
TGFB2	Transforming growth factor, beta 2	-4.92	B

OKAY: This gene's threshold cycle is reasonably low in both OV-VC and OV-KLK4 (<30). A: This gene's threshold cycle is relatively high (30-35) in either OV-VC or OV-KLK4, and is reasonably low in the other sample (<30). B: This gene's threshold cycle is relatively high (30-35) in both OV-VC and OV-KLK4.

The results from the 3 PCR array analyses (TGFβ, PAR and EMT) using mRNA from OV-KLK4 *vs.* OV-VC cells (Group 1) were compared with those from OV-KLK4-7 *vs.* OV-VC cells (Group 2). In total, 34 genes were found to be more than 2-fold differentially regulated in Group 1 and 74 genes in Group 2. Twenty-one genes overlapped within these 2 groups (**Table 16**). Of these 21 overlapping genes, 17 genes (80.9%) showed the same trend (either upregulation or downregulation) in both Group 1 and Group 2, however, COL1A2, COL5A2, KRT7 and MSN (all candidate genes which had also been analyzed by qPCR analyses) showed a strikingly different trend in the two groups (upregulation in one group and downregulation in the other). The overlapping genes also encompass further candidate genes (BMP5, JUNB, MMP1 and F10), which showed the same trend in the 2 groups.

It should be noted that all of the candidate genes, with exception of BMP5 (6.52) displayed rather low differential expression values in OV-KLK4 *vs.* OV-VC cells (<3.00). Still, with exception of KRT7 and F10, for all other genes differential expression was validated by independent qPCR analyses using different primers, probes, and mRNA preparations (see **4.12**).

Table 16. List of overlapping genes showing more than a 2-fold regulation in both OV-KLK4 and OV-KLK4-7 *vs.* OV-VC cells in the 3 PCR arrays

Gene symbol	Fold change		PCR array	Description
	OV-KLK4 *vs.* OV-VC (Group 1)	OV-KLK4-7 *vs.* OV-VC (Group 2)		
BMP5	6.52	20.25	TGFβ	Bone morphogenetic protein 5
CHRD	-3.80	-3.46	TGFβ	Chordin
COL1A2	-2.52	41.64	TGFβ	Collagen, type I, alpha 2
ID1	-5.80	-5.46	TGFβ	Inhibitor of DNA binding 1
ID2	-4.74	-5.50	TGFβ	Inhibitor of DNA binding 2
IL6	-2.09	-5.54	TGFβ	Interleukin 6
SMAD3	-2.06	-2.17	TGFβ	SMAD family member 3
EGR2	-2.20	-26.35	TGFβ	Early growth response 2
JUNB	-3.48	-12.13	TGFβ, PAR	Jun B proto-oncogene
MMP1	-6.96	-12.13	PAR	Matrix metallopeptidase 1
SERPINB5	-3.48	-3.03	PAR	Maspin
TNF	-3.48	-3.03	PAR	Tumor necrosis factor
TP53	2.30	2.64	PAR	Tumor protein p53
F10	2.30	10.56	PAR	Coagulation factor X
AHNAK	3.92	3.81	EMT	AHNAK nucleoprotein
CAMK2N1	-3.86	-7.26	EMT	Inhibitor of CAMKII
COL5A2	-2.69	71.01	EMT	Collagen, type V, alpha 2
DSP	2.71	2.58	EMT	Desmoplakin
KRT7	2.04	-1038.29	EMT	Keratin 7
MMP9	-3.68	-2.17	EMT	Matrix metallopeptidase 9
MSN	-2.89	396.18	EMT	Moesin

Red: upregulation; blue: downregulation; green: candidate genes further analyzed in the present work.

The PCR array results of Group 1 were also overall compared with those of Group 2 (**Figure 16**). Obviously, there were fewer genes differentially regulated in OV-KLK4 cells than in OV-KLK4-7 cells, and the fold change values of the regulated genes in OV-KLK4 cells were much smaller than those in OV-KLK4-7 cells (normalized to gene expression in OV-VC cells). Thus, KLK4 showed a much smaller impact on regulation of gene expression than KLK4-7 in OV-MZ-6 cells. KLK4 was originally thought to represent a major factor modulating gene regulation in OV-KLK4-7 cells, but these results do not support this hypothesis.

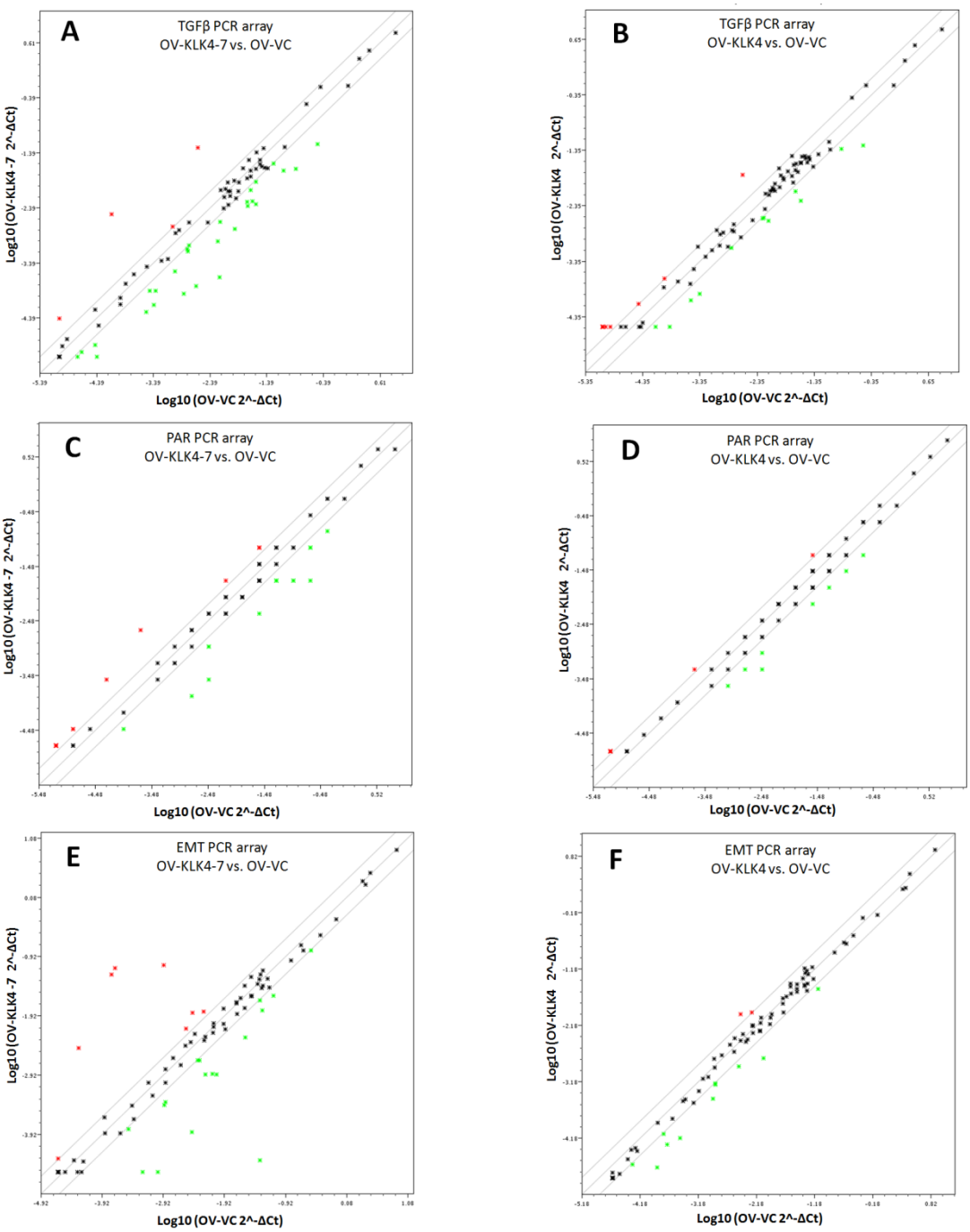

Figure 16. Comparison of the PCR array results of OV-KLK4 *vs.* OV-VC with those of OV-KLK4-7 *vs.* OV-VC cells

Each dot represents (at least) one gene from the PCR arrays. Values on X and Y axes reflect the genes' ΔCt values in the samples (ΔCt values from bottom/left to top/right: about 18 to about -4). Genes with Ct values of more than 35 were excluded. Black dots represent genes showing less than 2-fold regulation (less than 1.0 difference in ΔCt values) in OV-KLK4-7 *vs.* OV-VC and OV-KLK4 *vs.* OV-VC, respectively. Red/green dots represent genes showing more than a 2-fold upregulation/ downregulation. The distance of a dot from the central diagonal reflects the extent of differential regulation of the gene.

4.12 Comparison of the mRNA expression patterns of 10 candidate genes in six OV-MZ-6 cell lines by qPCR analysis

To further analyze the effects of overexpression of individual KLKs (KLK4, 5, 6 or 7) on gene expression in OV-MZ-6 cells, the mRNA expression levels of the ten previously selected candidate genes (**Table 11**) were characterized in the cell lines OV-KLK4, OV-KLK5, OV-KLK6 and OV-KLK7 (plus OV-VC and OV-KLK4-7) by qPCR analysis. The mRNA expression in OV-VC cells (set to 1.0 for each of the analyzed genes) was used as reference (**Figure 17**).

BMP4 mRNA was significantly downregulated in OV-KLK4-7 (0.12 ± 0.02, p=0.000) and OV-KLK5 (0.10 ± 0.02, p=0.000) cells. In OV-KLK4 cells, no significant difference of BMP4 mRNA expression was observed compared with OV-VC cells (1.65 ± 0.56, p=0.182). In contrast, significant upregulation of BMP4 mRNA was detected in OV-KLK6 (3.80 ± 0.27, p=0.003) and OV-KLK7 (2.98 ± 0.35, p=0.010) cells (**Figure 17A**).

BMP5 mRNA showed significant upregulation in all KLK-overexpressing cells: OV-KLK4-7 (12.64 ± 1.90, p=0.009), OV-KLK4 (4.55 ± 0.30, p=0.002), OV-KLK5 (21.89 ± 1.55, p=0.002), OV-KLK6 (5.97 ± 1.41, p=0.026) and OV-KLK7 (8.27 ± 2.58, p=0.008) cells (**Figure 17B**).

COL1A2 mRNA was significantly upregulated in OV-KLK4-7 (41.40 ± 4.22, p=0.004) cells. In OV-KLK5 cells, no significant difference of COL1A2 mRNA expression was observed compared with OV-VC cells (1.78 ± 0.39, p=0.074). In contrast, significant downregulation of COL1A2 mRNA was detected in OV-KLK4 (0.22 ± 0.01, p=0.000), OV-KLK6 (0.56 ± 0.06, p=0.005) and OV-KLK7 (0.38 ± 0.09, p=0.008) cells (**Figure 17C**).

COL5A2 mRNA showed significant upregulation in OV-KLK4-7 (32.37 ± 7.57, p=0.002) and OV-KLK5 (27.71 ± 3.89, p=0.007) cells. In OV-KLK7 cells, no significant difference of COL5A2 mRNA expression was observed compared with OV-VC cells (3.46 ± 1.39, p=0.092). In contrast, significant downregulation of COL5A2 mRNA was detected in OV-KLK4 (0.15 ± 0.04, p=0.001) and OV-KLK6 (0.25 ± 0.07, p=0.003) cells (**Figure 17D**).

KRT7 mRNA showed significant downregulation in OV-KLK4-7 (0.0010 ± 0.0001, p=0.000) and OV-KLK5 (0.0071 ± 0.0018, p=0.000) cells. In OV-KLK4 cells, no significant difference of KRT7 mRNA expression was observed compared with OV-VC cells (1.15 ± 0.07, p=0.069). In contrast, significant upregulation of KRT7 mRNA was detected in OV-KLK6 (4.01 ± 0.84, p=0.003) and OV-KLK7 (3.39 ± 0.72, p=0.029) cells (**Figure 17E**).

KRT19 mRNA showed significant upregulation in OV-KLK4-7 (240.71 ± 59.00, p=0.020) and OV-KLK5 (321.41 ± 113.74, p=0.008) cells. In OV-KLK6 (4.26 ± 2.27, p=0.130) and OV-KLK7 (1.15 ± 0.24, p=0.383) cells, no significant difference of KRT19 mRNA expression was observed compared with OV-VC cells. In contrast, significant downregulation of KRT19 mRNA was detected in OV-KLK4 (0.24 ± 0.09, p=0.005) cells (**Figure 17F**).

MSN mRNA showed significant upregulation in OV-KLK4-7 (280.75 ± 47.59, p=0.010) and OV-KLK5 (149.21 ± 43.55, p=0.028) cells. In OV-KLK6 (1.54 ± 0.25, p=0.066) and OV-KLK7 (1.19 ± 0.22, p=0.279) cells, no significant difference of MSN mRNA expression was observed compared with OV-VC cells. In contrast, significant downregulation of MSN mRNA was detected in OV-KLK4 (0.19 ± 0.04, p=0.001) cells (**Figure 17G**).

JUNB mRNA was significantly downregulated in all KLK-overexpressing cells: OV-KLK4-7 (0.09 ± 0.01, p=0.000), OV-KLK4 (0.20 ± 0.05, p=0.001), OV-KLK5 (0.05 ± 0.01, p=0.000), OV-KLK6 (0.08 ± 0.01, p=0.000) and OV-KLK7 (0.07 ± 0.03, p=0.000) cells (**Figure 17H**).

MMP1 mRNA showed significant downregulation in OV-KLK4-7 (0.11 ± 0.02, p=0.000) and OV-KLK4 (0.58 ± 0.14, p=0.006) cells. In OV-KLK7 (0.69 ± 0.14, p=0.058) cells, no significant difference of MMP1 mRNA expression was observed compared with OV-VC cells. In contrast, significant upregulation of MMP1 mRNA was detected in OV-KLK5 (5.44 ± 0.78, p=0.010) and OV-KLK6 (2.81 ± 0.73, p=0.013) cells (**Figure 17I**).

F10 mRNA was significantly upregulated in OV-KLK4-7 (9.86 ± 0.66, p=0.000) OV-KLK5 (17.84 ± 2.1, p=0.005) and OV-KLK7 (3.00 ± 0.31, p=0.008) cells. In contrast, significant downregulation of F10 mRNA was detected in OV-KLK4 (0.38 ± 0.03, p=0.001) and OV-KLK6 (0.26 ± 0.06, p=0.000) cells (**Figure 17K**).

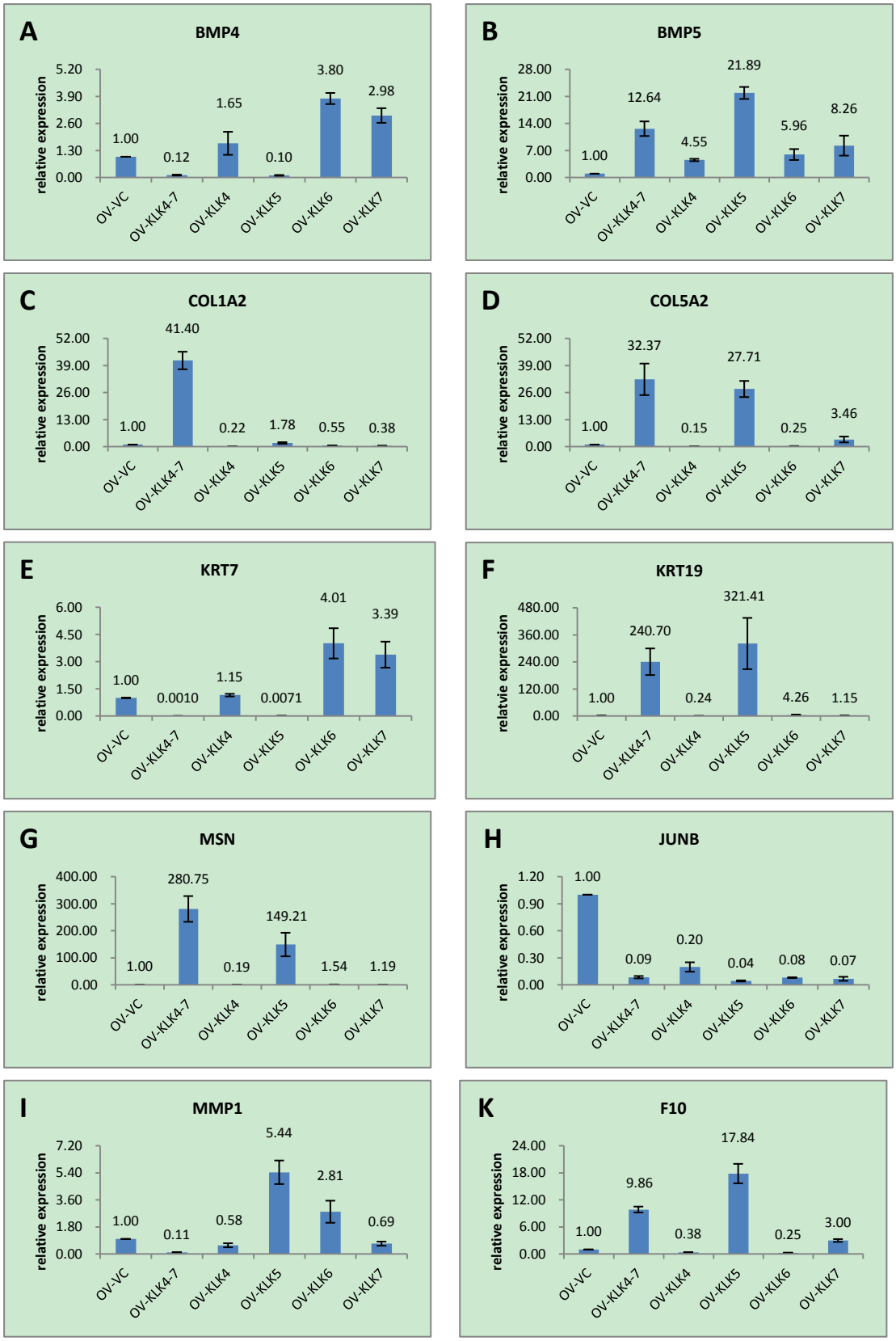

Figure 17. Comparison of the effects of overexpression of individual KLKs on gene expression of candidate genes in OV-MZ-6 cells by qPCR analysis

RNAs were extracted from OV-VC, OV-KLK4-7, OV-KLK4, OV-KLK5, OV-KLK6 and OV-KLK7 cells and qPCR analysis was performed to compare the mRNA

expression levels of the 10 candidate genes (**Table 11**) in the 6 different cell lines. The relative expression levels of the genes are depicted with OV-VC as the reference (expression level set to 1.0). Eight out of 10 candidate genes, *i.e.* BMP4, BMP5, COL5A2, KRT7, KRT19, MSN, JUNB and F10, are similarly regulated in OV-KLK5 and OV-KLK4-7 cells.

In summary we found that in 8 out of the 10 analyzed candidate genes, *i.e.* in BMP4, BMP5, COL5A2, KRT7, KRT19, MSN, JUNB and F10, gene expression was regulated in OV-KLK5 cells in a similar manner as in OV-KLK4-7 cells. COL1A2 mRNA showed no significant difference in OV-KLK5 cells, but significant downregulation in other 3 individual KLK cell lines. Concerning MMP1 mRNA regulation, OV-KLK5 cells showed significant upregulation, while OV-KLK4-7 cells showed significant downregulation. Two genes were found to be significantly upregulated (BMP5) or downregulated (JUNB) in all cell lines overexpressing one or more of the KLKs (OV-KLK4, OV-KLK5, OV-KLK6, OV-KLK7, and OV-KLK4-7). All in all, the obtained results indicate that KLK5 is the predominant regulator of gene expression in OV-KLK4-7 cells.

4.13 Analysis of the protein expression of selected candidate genes in OV-MZ-6 cell lines individually expressing either KLK4, 5, 6 or 7 by Western blot analysis

In **Results 4.6**, we had selected 4 of the 10 candidate genes (MSN, KRT19, KRT7 and JUNB) for further expression analysis in OV-KLK4-7 *vs.* OV-VC cells on the protein level. Additionally, we also performed Western blot analysis in OV-KLK4, OV-KLK5, OV-KLK6 and OV-KLK7 cells (**Figure 18**). In line with the previous results, a clear upregulation of MSN and KRT19 and downregulation of KRT7 was observed in OV-KLK4-7 cells compared with OV-VC cells. Only OV-KLK5 cells showed similar protein expression levels of MSN, KRT19 and KRT7 as OV-KLK4-7 cells, which was in consistence with the qPCR results (**Figure 17G, F and E**). With respect to JUNB, all 5 cell lines showed (a slight) downregulation compared with OV-VC cells which was also consistent with qPCR results (**Figure 17H**). These results support the notion that KLK5 exerts the strongest impact on protein expression in OV-KLK4-7 cells.

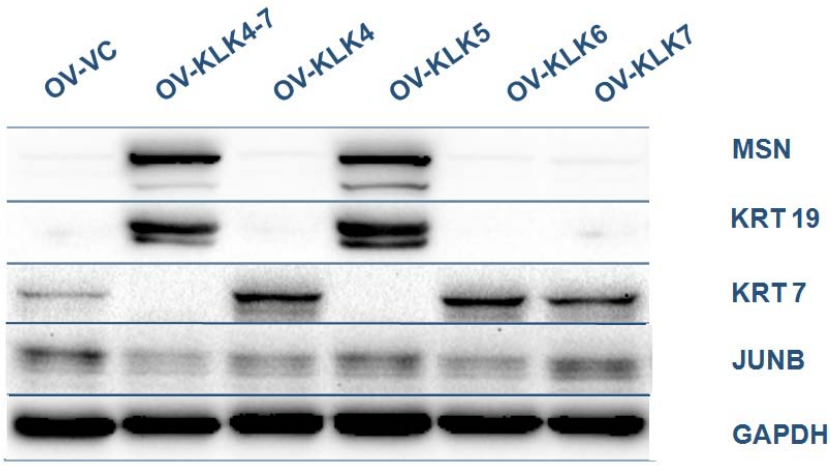

Figure 18. Comparison of protein expression levels of several candidate genes in different OV-MZ-6 cell lines by Western blot analysis
Protein samples (40 µg/lane) were extracted from cells and Western blot analysis of MSN, KRT19 and KRT7 protein expression in OV-VC, OV-KLK4-7, OV-KLK4, OV-KLK5, OV-KLK6 and OV-KLK7 cells was performed. Only KLK5-expressing cells showed a similar protein expression pattern concerning MSN, KRT19 and KRT7 compared with OV-KLK4-7 cells. GAPDH was used as the reference.

4.14 Analysis of the effects of a specific KLK5 inhibitor on gene regulation in OV-MZ-6 cells

The previous results demonstrated that KLK4-7 overexpression regulates gene expression in OV-MZ-6 cells and that KLK5 may exert the strongest impact on gene regulation in these cells. To test whether enzymatic activity of KLK5 is necessary for mediating these effects, we aimed at selectively inhibiting KLK5 activity in the cellular system. For this purpose a KLK5-specific inhibitor, SFTI-FCHR Y7N14, recently developed by J. Harris and co-workers (Queensland University of Technology, Australia) was used. This inhibitor is derived from the sunflower trypsin inhibitor (SFTI). Firstly, the cytotoxicity of the inhibitor was analyzed in proliferation assays using KLK5-deficient OV-VC cells. Here, no effects were observed on cell proliferation by this inhibitor applied in two different concentrations (0.5 and 2.0 µM) (**Figure 19A**). Next, three cell lines (OV-VC, OV-KLK5, and OV-KLK4-7) were cultured with/without this inhibitor (2.0 µM) for 4 days (with a change of medium plus inhibitor after 2 days) and cell samples were collected. Expression of six

candidate genes (COL1A2, COL5A2, JUNB, MSN, KRT19 and KRT7) as well as KLK5 was analyzed in the samples by qPCR. If enzymatic activity of KLK5 is necessary and the inhibitor SFTI-FCHR Y7N14 would be efficient in the cellular system, one would expect that expression of COL1A2, COL5A2, KRT19, and MSN will be reduced in KLK4-7- and KLK5-overexpressing cells, whereas KRT7 and JUNB should be upregulated.

Samples without inhibitor treatment were used as reference (with the gene expression level set to 1.0). Surprisingly, all cells which were treated with the inhibitor showed a significant downregulation of JUNB expression (OV-VC 0.41 ± 0.01 p=0.000, OV-KLK5 0.11 ± 0.01 p=0.000, and OV-KLK4-7 0.23 ± 0.02 p=0.000, respectively).

In OV-VC cells, only minute effects of the inhibitor on expression of the other genes were observed (less than 1.5-fold difference, **Figure 19B**). OV-KLK5 cells showed significant downregulation of KRT19 expression (0.43 ± 0.09 p=0.007) and upregulation of KLK5 (2.81 ± 0.32 p=0.010) after inhibitor treatment (**Figure 19C**). OV-KLK4-7 cells showed also significant downregulation of KRT19 (0.47 ± 0.09 p=0.009), but here KLK5 expression was reduced as well (0.58 ± 0.12 p=0.028) after inhibitor treatment (**Figure 19D**). These results indicate that this inhibitor has some effects on expression of some genes in OV-MZ-6 cells; however, the observed effects on one hand are not very strong and on the other may not be related to inhibition of KLK5. Thus, further studies are necessary to unravel the effects of the inhibitor SFTI-FCHR Y7N14 on gene expression and, furthermore, to evaluate the role of enzymatic activity of KLK5 in regulation of gene expression in ovarian cancer cells.

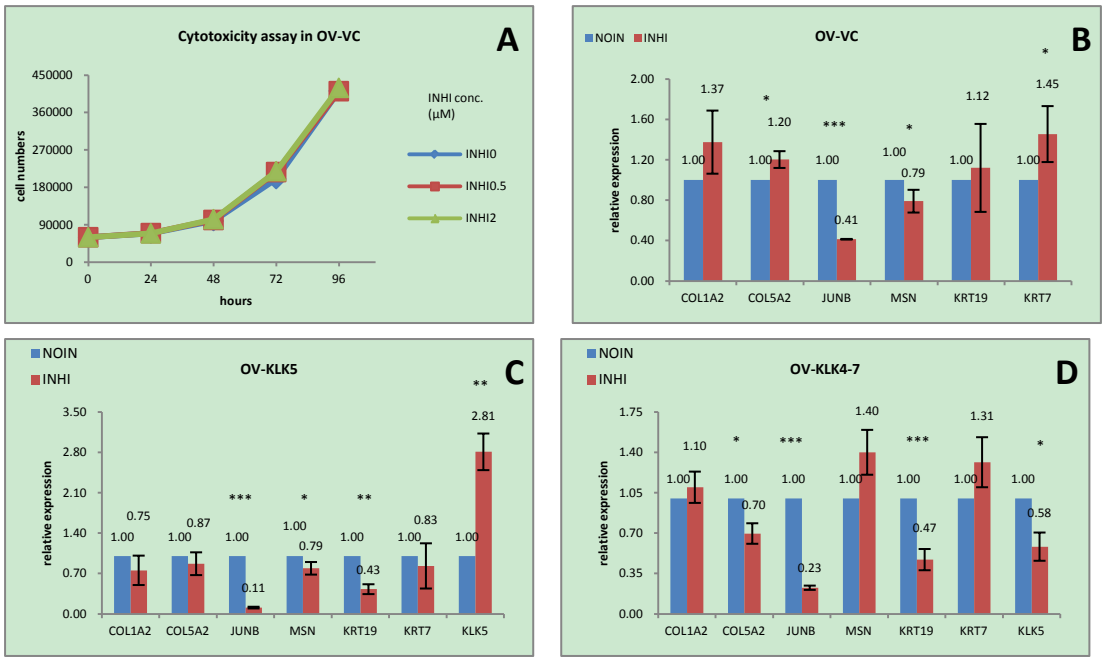

Figure 19. Effects of a KLK5-specific inhibitor (SFTI-FCHR Y7N14) on gene regulation in OV-MZ-6 cells

A: The SFTI-derived KLK5-specific inhibitor showed no obvious effects on proliferation of OV-VC cells in two concentrations (0.5 and 2.0 μM). B: OV-VC cells showed significant upregulation of COL5A2 and KRT7 and downregulation of JUNB and MSN following inhibitor treatment. C: OV-KLK5 cells showed significant upregulation of KLK5 and downregulation of JUNB, MSN and KRT19 following inhibitor treatment. D: OV-KLK4-7 cells showed significant downregulation of COL5A2, JUNB, KRT19 and KLK5 after treatment with the inhibitor. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

5 Discussion

5.1 Overexpression of KLK4-7 regulates expression of other cancer-related genes in OV-MZ-6 ovarian cancer cells

By PCR array screening, expression of many genes was found to be modulated in KLK4-7-overexpressing *vs.* vector control OV-MZ-6 ovarian cancer cells. As summarized in **Table 11**, some genes, particularly MSN, KRT7, KRT19, and JUNB, displayed a distinct differential expression pattern. These findings were further confirmed by qPCR, Western blot, immunofluorescence (IF), and immunohistochemistry (IHC) analyses. All the results, thus, demonstrate that overexpression of KLK4-7 significantly regulates expression of other cancer-related genes in OV-MZ-6 cells.

Shahinian and co-workers [173] have analyzed the proteomic profile of cell-conditioned medium (CCM) of OV-KLK4-7 *vs.* OV-VC cells using mass spectrometry. In that study, 45 secreted proteins were detected which were significantly up- or down-regulated. In addition, 39 of these significantly affected proteins were increased and only 6 were decreased in abundance. These data suggest that overexpression of KLK4-7 does not result in major degradation, but - considering the proposed function of the identified differentially regulated proteins - rather appears to regulate cell-cell communication and to increase TGFβ1 signaling in the ovarian cancer cells. This report was the first to analyze the effects of overexpression of KLK4-7 on protein expression in OV-MZ-6 cells. Different from that report, the present project predominantly focused on analyzing the effects of overexpression of KLK4-7 on gene expression (mRNA) in OV-MZ-6 cells before validation on the protein level. Interestingly, three genes identified to be regulated on the mRNA level were also found in distinctly different amounts in the CCM on the protein level in the earlier report [173]: COL5A2 (>30-fold in CCM of OV-KLK4-7 *vs.* OV-VC cells), KRT19 (>8-fold), and MSN (>7-fold) (O. Schilling, personal communication). However, moesin (MSN) and keratin 19 (KRT19) are not annotated as secreted proteins. The presence of these proteins in the CCM may result from increased cell death during cultivation of the cells in serum-free medium to obtain the CCM. Still, the presence of moesin and keratin 19 in significantly higher amounts in CCM derived from OV-KLK4-7 *vs.* OV-VC cells strongly supports the finding concerning

differential expression in these two cell lines.

Our collaborators from the Queensland University of Technology in Brisbane analyzed differential gene expression in OV-KLK4-7 *vs.* OV-VC cells by the Agilent Human Genome CGH Microarray technique. The *Top 10* upregulated and downregulated genes in OV-KLK4-7 *vs.* OV-VC cells found in their study are shown in **Appendix 10.4** (D. Loessner, personal communication)**.** Two genes identified in the present project, KRT19 and MSN, were among the *Top 10* of the highest differentially upregulated genes in their study (# 2 and # 3, respectively). Another gene, COL1A2, is listed as # 22 of the upregulated genes in OV-KLK4-7 *vs.* OV-VC cells. KRT7 is among the highest differentially downregulated genes in OV-KLK4-7 *vs.* OV-VC cells (# 4). Around 20,000 genes were analyzed in their study, while we analyzed only about 240 genes from three different pathway-related PCR arrays. Comparing the overlapping genes of these two independent studies, the results display strong consistency, thus, strongly supporting the results in the present study. Furthermore, the analysis using pathway-related low-density microarrays proved to be efficient not only in selection of differentially regulated genes in OV-KLK4-7 *vs.* OV-VC cells but also in underlining the involvement of relevant pathways, especially concerning genes involved in EMT and TGFβ signaling.

In order to better understand the possible tumor-related functions of the genes (and gene products) which expression is modulated by KLK4-7 overexpression, the following paragraphs give an overview of the known possible functions of the ten selected genes (**Table 11**), which were further analyzed in the present study.

Moesin is a membrane-organizing extension spike protein, which belongs to the small ERM protein family encompassing three homologous members: ezrin, radixin, and moesin. Moesin functions as a cross-linker between the plasma membrane and the cytoskeleton and is important for cell-cell recognition, signaling and cell movement. Köbel and co-workers [176] found that ezrin and moesin showed strong expression in 49% and 48% of ovarian cancer samples respectively and their expression correlated with reduced overall survival. Furthermore, ERM proteins were found to be involved in multiple aspects of cell migration and high expression of moesin was positively correlated with cancer progression [177]. In the present study, a strong upregulation of moesin on both mRNA and protein level was confirmed in OV-KLK4-7 *vs.* OV-VC ovarian cancer cells. This finding may indicate that upon overexpression of KLK4-7

an increased moesin expression results in a more malignant phenotype of the tumor cells. In addition to the results obtained within the cell biological analyses, moesin expression was also analyzed by immunohistochemistry in human tissues. Here, high expression of moesin was demonstrated in ovarian cancer tissue, whereas there was no staining at all in normal ovary tissue. Moreover, the staining intensity of moesin seemed to be correlated with tumor stages (**Figure 14**).

Keratins such as keratin 7 (KRT7) and keratin 19 (KRT19) are intermediate filament proteins responsible for the structural integrity of epithelial cells. KRT7 can be used as a marker to distinguish ovarian cancer from colonic cancer (positive in 90.8% ovarian cancer and negative in 82.9% colon cancer) [178]. Similarly, KRT7 was also used to distinguish renal cancer from ovarian cancer. A study found that 14 out of 14 ovarian clear cell carcinomas were positive for KRT7 with intense cytoplasmic and membranous reactivity whereas only 2 of 17 renal clear cell carcinomas showed focal membranous reactivity [179]. KRT19 is also known as CYFRA 21-1. Gadducci and co-workers [180] found that preoperative serum CYFRA 21-1 levels were significantly higher in patients with ovarian cancer compared with those with benign ovarian disease. Besides, preoperative CYFRA 21-1 levels were significantly lower in the patients, who achieved a pathologic complete response compared with those, who had clinically or surgically detectable disease after chemotherapy. Therefore, the high preoperative serum CYFRA 21-1 levels were associated with chemoresistance of ovarian cancer patients. Another study found that a high portion of ovarian cancer stem cells with CD44 and KRT19 co-expression in epithelial ovarian tumors correlated with poor progression-free survival of patients [181]. In our study, a strong upregulation of KRT19 and downregulation of KRT7 was found in OV-KLK4-7 *vs.* OV-VC cells. Indicated by the previous reports [180,181], KRT19 expression was positively associated with resistance of ovarian cancer patients to chemotherapy and poor prognosis. Thus, upregulation of KRT19 induced by KLK4-7 overexpression could increase the malignancy of OV-MZ-6 cells and be an explanation for the report from Loessner *et al.* that OV-KLK4-7 cells were more insensitive to paclitaxel treatment compared with OV-VC cells [171].

Jun proteins (c-Jun, JunB, and JunD) play an important role in the regulation of cell proliferation, apoptosis, and angiogenesis and display a strong impact on carcinogenesis and progression of several tumor types. Together with Fos proteins,

they form several different types of homo- and heterodimers, named AP-1 transcription factor [182]. In combination with c-Fos, c-Jun expression supports tumorigenesis, whereas JunB is an inhibitor of cell division, an inducer of senescence and a tumor suppressor [183-186]. In the present study, JunB showed downregulation in OV-KLK4-7 *vs.* OV-VC cells. Downregulation of JunB modulated by KLK4-7 overexpression may, thus, increase cell proliferation and promote tumorigenesis of ovarian cancer cells.

Type I collagen is the most abundant human collagen which is composed of three chains, two alpha1(I) chains and one alpha2(I) chain. Type I collagen, by regulating the integrin-PTEN/PI3K/Akt signaling pathway in remodeling ECM, strongly enhances metastasis/invasion of ovarian cancer cells [187]. Cheng and Leung found that type I collagen could downregulate E-cadherin expression and induce the epithelial-mesenchymal transition (EMT) in SKOV3 ovarian cancer cells and PC3 prostate cancer cells [188]. Type V collagen is associated with classical Ehlers-Danlos syndrome which is caused by a defect in the structure, production or processing of collagen or proteins that interact with collagen. One study showed that type V collagen promoted the malignant phenotype of various pancreatic cancer cell lines by increasing cell adhesion, migration and viability [189]. Upregulation of collagens (as indicated by increased expression of COL1A2 and COL5A2 in OV-KLK4-7 *vs.* OV-VC cells) could induce the EMT process and promote the invasion/metastasis of ovarian cancer cells which may explain the more malignant phenotype of OV-KLK4-7 cells observed by Prezas *et al.* and Loessner *et al.* [170-172].

Matrix metalloproteinase-1 (MMP1) is known as an interstitial collagenase and fibroblast collagenase which is involved in the breakdown of extracellular matrix. MMP-1 cleaves the interstitial collagens type I, II, and III. Downregulation of MMP1, which breaks down collagen I, may further strengthen the effect of collagen I upregulation as mentioned above. F10 is coagulation factor X. Wang and co-workers [190] found that coagulation factors exhibited tumorigenic functions, including regulation of tumor cell proliferation, angiogenesis, invasion, and metastasis. Factor Xa (the activated form of factor X) can activate PAR1, 2, and 3 signaling [191-193], which further regulates the downstream proteins. By this way, KLK4-7 could promote proliferation and metastasis of ovarian cancer cells.

The bone morphogenetic protein (BMP) family is a member of the TGFβ super family.

Laatio *et al*. [194] propose that strong expression of BMP4 before chemotherapy correlated with better progression-free and overall survival of ovarian cancer patients. Another study demonstrated that BMP4 suppressed cell growth both *in vitro* and *in vivo* [195], whereas downregulation of BMP4 might increase proliferation and metastasis of cancer cells [196,197]. We found a significant downregulation of BMP4 in KLK4-7-overexpressing OV-MZ-6 cells. Therefore, by downregulation of BMP4, overexpression of KLK4-7 may support tumor growth and metastasis of ovarian cancer cells. In contrast to BMP4, BMP5 has, so far, not been intensively analyzed for its tumor biological role.

Overexpression of KLK4-7 significantly increases the malignancy of ovarian cancer cells [169-173]. The present study shows that overexpression of KLK4-7 distinctly regulates the expression of other genes in OV-MZ-6 ovarian cancer cells, which are strongly associated with tumorigenesis and progression of ovarian cancer as indicated by the reports mentioned above.

By comparison of the three PCR array results, we found that KLK4-7 strongly regulate the expression of genes encoding proteins involved in EMT and the TGFβ pathway, respectively. The proteins encoded by three of the identified genes, MSN, KRT7 and KRT19 (all EMT genes) are structurally and functionally closely related: moesin links cell membrane and cytoskeleton (keratins are components of cytoskeleton); moesin and keratins are responsible for cell integrity and movement which is an important part of the EMT process. This fact suggests that KLK4-7 overexpression increases the malignancy of OV-MZ-6 cells probably by promoting the EMT process. With the indication of EMT in OV-KLK4-7 cells, it is tempting to assume that the biological behavior of OV-KLK4-7 cells may be altered as well. However, migration assays showed no obvious difference between OV-KLK4-7 and OV-VC cells. In previous experiments [170], neither the proliferation rate nor the adhesive capacity of OV-KLK4-7 cells were different from that of OV-VC cells in the monolayer cell culture experiments. Only in Matrigel invasion assays a significantly increased invasive behavior of OV-KLK4-7 *vs.* OV-VC cells was observed. In *in vivo* experiments, a strong impact of KLK4-7 expression was observed on tumor growth and spread [170-172]. Thus, standard monolayer-based proliferation, adhesion, and migration assays may not be suitable to monitor changes in the tumor-relevant biological behavior of KLK4-7-overexpressing OV-MZ-6 cells.

In order to analyze differential gene expression in OV-KLK4-7 *vs.* OV-VC cells in a model system more closely resembling the tumor microenvironment, a novel 3-dimensional (3D) cell culture model based on gelatin methacryloyl (GelMA) hydrogel was established in our laboratories in collaboration with D. Loessner and D. Hutmacher (QUT, Brisbane). Here, OV-VC and OV-KLK4-7 cells formed spheroids inside the hydrogel. However, RNA could not be isolated in sufficient yields and quality from the spheroids, which may be due to (i) the relatively low cell numbers in the spheroids and (ii) the presence of (proteinous) impurities in the isolated RNA derived from the cells embedded in the hydrogel matrix. Other matrices, such as non-proteinous polyethylene glycol-based hydrogels [198], may be more suitable for isolation of RNA. Still, concerning the size of the spheroids, OV-KLK4-7 cells grew to larger spheroids than OV-VC cells. In a previous study, Loessner *et al.* [172] cultured OV-VC and OV-KLK4-7 cells in the same 3D model (GelMA) and found that the proliferation of OV-KLK4-7 was significantly enhanced compared to vector controls. Thus, the results shown in the present study are in line with the previous report. Although the established 3D model is not suitable to obtain high quality RNA, this relatively inexpensive 3D cell culture model can be applied *e.g.* for analysis of the effectiveness of ovarian cancer treatments in the future.

5.2 KLK5 exerts the strongest impact on gene regulation in OV-KLK4-7 cells

The results discussed above demonstrate that overexpression of KLK4-7 strongly regulates expression of other cancer-related genes in OV-MZ-6 cells. To evaluate whether co-expression of all four KLKs is a prerequisite for the observed effects on gene regulation or whether individual overexpression of one of these KLKs is sufficient for mediating these effects, single-KLK-transfected OV-MZ-6 cells were analyzed. Based on publications of the strong impact of KLK4 on modulation of tumor-relevant processes [33,140,199,200], the initial hypothesis focused on KLK4 being distinctly responsible for the effects of gene regulation in OV-KLK4-7 cells. Therefore, the three different PCR array analyses (analyzing genes involved in EMT, TGFβ and PAR-signaling, respectively) were also performed for OV-KLK4 cells and compared to the gene expression patterns in OV-VC cells. However, KLK4 showed much smaller effects on gene regulation than KLK4-7 in OV-MZ-6 cells (**Figure 16**). Of the 34 genes regulated in OV-KLK4 *vs.* OV-VC cells, 21 genes overlapped with

those in OV-KLK4-7 *vs.* OV-VC cells (**Table 16**). Among these 21 overlapping genes, 17 genes showed the same trend of regulation (either upregulation or downregulation). However, COL1A2, COL5A2, KRT7, and MSN (all selected candidate genes, **Table 11**) showed a strikingly different regulation trend (**Table 16**). Therefore, this hypothesis was not fully supported.

Subsequently, we focused on analyzing the expression of ten selected candidate genes (**Table 11**) in OV-VC, OV-KLK4, OV-KLK5, OV-KLK6, OV-KLK7, and OV-KLK4-7 cells. Here, the results indicated that KLK5 exerted the strongest impact on gene regulation, for example on expression of MSN, KRT7, and KRT19, in OV-KLK4-7 cells (**Figures 17, 18**).

Dorn *et al.* [201] reported that KLK5 levels were significantly higher in the serum of ovarian cancer patients compared to those with benign ovarian tumors. Besides, elevated KLK5 level in serum and ascitic fluid of ovarian cancer patients was correlated with poor progression-free survival. In line with this, Yousef and co-workers [76] found higher concentrations of serum KLK5 in 69% of ovarian cancer patients, whereas KLK5 is almost not detectable in serum of normal individuals. High levels of KLK5 were also observed in tissue extracts and ascitic fluid from metastatic ovarian cancer patients [76]. Another study also showed high serum KLK5 levels in 52% of patients with ovarian cancer [202].

Furthermore, a significant positive correlation between KLK5 expression and tumor grade 3 and disease stages III/IV was observed in ovarian cancer patients [141,144]. Survival analysis revealed that ovarian cancer patients positive for KLK5 expression had a significantly increased risk for relapse and death [141,144]. High KLK5 expression also showed an independent prognostic value for poor progression-free survival in the subset of lower grade (grades 1 and 2) ovarian cancer patients [141]. Another study supported the notion that KLK5-positive ovarian cancer patients had a significantly shorter progression-free survival and overall survival [144]. In a recent study, KLK5 protein expression (measured by ELISA) was found significantly correlated with advanced age and high nuclear grade (grade 3). It should be noted, however, that patients with elevated stromal cellular KLK5 expression displayed a significantly lower risk of recurrence as compared to patients with low stromal cellular KLK5 expression in the subgroup of FIGO III/IV ovarian cancer [203]. The discrepancy between these reports could be due to the fact that different patient

cohorts and different parts of tissue (cancer cells or stromal cells) were analyzed.

A series of splice variants have been detected within the KLK gene family [204]. Kurlender *et al.* [35] found that the KLK5 splice variant KLK5-SV1 was observed in 9 out of 10 ovarian cancer tissues, but was not detected in normal ovarian tissue. Another KLK5-splice variant, KLK5-SV2, was not detectable in normal ovarian tissue. However, the expression of KLK5-SV2 was at relatively high levels in 10 of 20 ovarian cancer tissues and at low levels in 4 cases. High expression levels of KLK5-SV2 were also found in CAOV-3 ovarian cancer cell line [36]. In another study, Dong and co-workers [34] characterized a novel KLK5 transcript with a short 5'-untranslated region (UTR) (this splice variant is similar to KLK5-SV1, but lacks 32 bp in the 5'-UTR and a G nucleotide at the end of the first non-coding exon) as well as a novel KLK7 transcript with a long 3'-untranslated region. Both mRNA variants showed high expression in the ovarian cancer cell lines OVCAR-3 and PEO1, respectively, but were expressed at low levels in normal ovarian epithelial cells [34]. The above mentioned studies showed that high levels of KLK5 splice variants were found in ovarian cancer tissues and cell lines, indicating their potential to serve as biomarkers of ovarian cancer.

All reports mentioned above demonstrate: (i) KLK5 (including KLK5 splice variants) showed high expression in ovarian cancer samples and could be used as detection biomarkers for ovarian cancer; (ii) KLK5 expression was found in most cases to be correlated with poor prognosis of ovarian cancer patients; and (iii) KLK5 was associated with more aggressive forms of ovarian cancer probably by promoting tumor progression.

Yet, it is not fully elucidated how KLK5 is associated with more aggressive forms of ovarian cancer. Previous studies suggest that KLK5, by degrading the extracellular matrix components collagens type I, II, III, and IV, fibronectin, and laminin, affects the invasion and metastasis of ovarian cancer cells [144,202]. Furthermore, KLK5 can potentially release (i) angiostatin 4.5 (consisting of plasminogen kringle 1-5) from plasminogen, (ii) "cystatin-like domain 3" from low molecular weight kininogen, and (iii) fibrinopeptide B and peptide beta15-42 from the Bbeta chain of fibrinogen. KLK5 could also regulate the binding of plasminogen activator inhibitor 1 to vitronectin. These findings suggested that KLK5 might be involved in tumor progression, invasion and angiogenesis of ovarian cancer [205]. Similarly, other

studies indicated that KLK5 increased the invasiveness and metastasis of urinary bladder carcinoma and oral squamous cell carcinoma by its degradative and cleavage activity [206,207].

However, in addition to the direct cleavage of ECM proteins, our study strongly indicates that KLK5 may act as a signaling molecule affecting tumor-related genes. Interestingly, some recent studies suggest that KLK5 could strongly suppress the malignancy of breast cancer cells by regulation of signaling or pathways [208,209]. Pampalakis and co-workers [208] found that re-expression of KLK5 in MDA-MB-231 breast cancer cells decreased malignancy *in vitro* and *in vivo* dose-dependently. Reactivation of KLK5 inhibited key EMT genes. The authors suggest that KLK5 might suppress breast cancer by regulating the mevalonate pathway. Besides, KLK5 could also play a role in regulating miRNA networks, which further affect MMPs and other extracellular matrix (ECM) targets. Thus, KLK5 could activate the miRNA-mediated anti-oncogenic pathways in breast cancer [209]. These studies showing that KLK5 regulates several EMT genes and miRNA networks in breast cancer are supportive to our findings that KLK5 strongly regulates other cancer-related genes, such as EMT- and TGFβ-signaling-associated genes, in OV-MZ-6 ovarian cancer cells. The difference of KLK5 on promoting or suppressing malignancy of cancer cells could be due to the fact that in different types of cancers, KLK5 targets a different substrate repertoire leading to activation of either tumor-supporting or –suppressing signaling pathways.

Besides, the KLK5-specific inhibitor SFTI-FCHR Y7N14 was used to selectively inhibit KLK5 activity in OV-KLK4-7 and OV-KLK5 cells. The expression profile of selected genes in cells with/without treatment of the inhibitor indicates that this inhibitor affects expression of some genes in OV-MZ-6 cells. However, the observed effects are not very strong and may even not be related to inhibition of KLK5. Thus, further studies are necessary to unravel the effects of the inhibitor on gene expression and, furthermore, to evaluate the role of enzymatic activity of KLK5 in regulation of gene expression in ovarian cancer cells. As KLK5 could be a potential therapeutic target, the research on KLK5 inhibitors may lead to findings of effective treatments for ovarian cancer patients in the future.

There are also reports of KLK4 [33,140,210], KLK6 [143,145-151] and KLK7 [34,152,153,211] in ovarian cancer. Similar to KLK5, KLK4, 6, and 7 were found in

elevated levels in ovarian cancer tissue and cell lines [33,34,143,145,152]. A significant positive correlation between KLK4 expression and tumor grade and clinical stage was observed [140]. Furthermore, patients afflicted with ovarian cancer positive for KLK4 expression showed an increased risk for relapse and death [140]. Besides, KLK4 was found to induce paclitaxel resistance in ovarian cancer [210,212]. Similarly, serum KLK6 concentration was found higher in patients with late-stage, higher-grade ovarian cancer [150]. KLK6 expression was a poor prognostic marker for progression-free and overall survival [151]. Another study showed that KLK7 expression levels were clearly higher in patients with grade 3 than those with grade 1 to 2 ovarian cancer. KLK7 high expression was correlated with poor disease-free and overall survival of patients with low grade (grade 1 to 2) ovarian cancer [153]. In addition, KLK7 was also found to promote paclitaxel resistance in serous epithelial ovarian cancer [211]. The reports mentioned above suggest that KLK4, 6, and 7 are also associated with a more malignant phenotype of ovarian cancer. Although KLK4, 6, and 7 do not have a major impact on gene regulation in OV-MZ-6 cells, they together with KLK5 might increase cell malignancy in a much stronger way than that of any individual KLK alone.

6 Conclusion and Outlook

In this study, a significant upregulation of MSN, KRT19, COL1A2, COL5A2, BMP5, and F10, as well as significant downregulation of KRT7, JUNB, BMP4, and MMP1 was observed in KLK4-7-overexpressing compared to vector-control OV-MZ-6 ovarian cancer cells. These results demonstrate that KLK4-7 have a very strong regulatory impact on gene/protein expression in OV-MZ6 cells, especially on EMT- and TGFβ-associated genes. This finding distinctly expands the common view that KLKs promote cancer progression mainly by their degradative pericellular proteolysis function. KLKs may also regulate tumor invasion and metastasis by acting as signaling molecules promoting activation of the EMT process and/or TGFβ signaling. KLK5 presumably functions as the major gene regulator in OV-KLK4-7 cells.

In the future, further analysis of the correlation between some KLKs or regulated genes (*e.g.* moesin) and clinicopathological features may lead to the identification of promising biomarkers for diagnosis and/or prognosis of ovarian cancers. Furthermore, analysis of inhibitors of KLKs may result in effective treatments for ovarian cancer patients.

7 Summary

In ovarian cancer, the most critical clinical problem is high mortality due to late diagnosis. Recently, more and more reports link the family of kallikrein-related peptidases (KLKs), especially KLK4, 5, 6, and 7 (KLK4-7), to malignancy and tumor progression of ovarian cancer. Overexpression of KLK4-7 promotes tumor metastasis and increases paclitaxel chemoresistance of ovarian cancer cells. The present project aimed at analyzing the effects of overexpression of KLK4-7, as well as individual KLK4, 5, 6, or 7, on gene regulation in OV-MZ-6 ovarian cancer cells.

In this study, three PCR arrays (TGFβ [transforming growth factor beta], PAR [protease activated receptor], and EMT [epithelial-mesenchymal transition]) were used to screen for differentially expressed genes in KLK4-7-overexpressing (OV-KLK4-7) *vs.* vector-control (OV-VC) OV-MZ-6 cells. Based on the screening results, ten genes (BMP4, BMP5, COL1A2, COL5A2, KRT7, KRT19, MSN, JUNB, MMP1, and F10) were selected as candidate genes for further analysis. Validation of differential gene/protein expression of the ten candidate genes was performed by qPCR, Western blot, immunofluorescence, and immunohistochemical analyses. On the mRNA level, BMP5, COL1A2, COL5A2, KRT19, MSN, and F10 showed significant upregulation, BMP4, KRT7, JUNB, and MMP1 significant downregulation in OV-KLK4-7 *vs.* OV-VC cells. Strong upregulation of MSN and KRT19 as well as downregulation of KRT7 and JUNB in OV-KLK4-7 *vs.* OV-VC cells was confirmed also on the protein level.

In the second part of this project, the mRNA and protein expression of candidate genes was compared in the five KLK-transfected and vector-control OV-MZ-6 cell lines (OV-VC, OV-KLK4-7, OV-KLK4, OV-KLK5, OV-KLK6 and OV-KLK7) by qPCR and Western blot analyses respectively. Seven out of the ten candidate genes, *i.e.* BMP4, BMP5, COL5A2, KRT7, KRT19, MSN, and F10, only OV-KLK5 cells showed a similar mRNA expression pattern as compared to OV-KLK4-7 cells. On the protein level, only OV-KLK5 cells showed similar expression of MSN, KRT19, and KRT7 as OV-KLK4-7 cells. Another candidate gene JUNB showed downregulation in all five KLK-transfected cell lines compared with OV-VC cells on both mRNA and protein level.

In conclusion, the significant upregulation of MSN and KRT19 as well as downregulation of KRT7 and JUNB both on the mRNA and protein level in KLK4-7-overexpressing OV-MZ-6 ovarian cancer cells is reported for the first time. The results indicate that overexpression of KLK4-7 significantly regulates gene expression in ovarian cancer cells and KLK5 exerts the strongest impact on gene regulation in OV-KLK4-7 cells. Further analysis of the individual KLKs and regulated genes may lead to the findings of useful diagnosis/prognosis biomarkers and effective treatments for ovarian cancer.

8 Acknowledgement

First of all, I would like to thank my supervisor, Prof. Dr. rer. nat. Viktor Magdolen for providing me the opportunity to work on this interesting project. Whenever I had problems in the lab, he always tried his best to help me and discussed them with me in detail. Without his great guidance, I could never finish the project. In the beginning of my life in Munich, I encountered many troubles and he helped me a lot. I will always remember this and I am really grateful for what he has done for me.

I would like to thank my second supervisor, Dr. Christof Seidl for his helpful guidance throughout the project. He is always willing to give me the suggestions and advices, especially on my presentations and dissertation writing.

I would like to also express my gratitude to: Prof. Dr. rer. nat. Manfred Schmitt; Prof. Dr. rer. nat. Ute Reuning; Claudia Beutner; Sabine Creutzburg for her help with my Western blot experiments; Anke Benge and Elisabeth Schueren for their help with my cell experiments; Daniela Hellmann for her help with the IHC experiments; Rudolf Napieralski for his helpful discussions.

I would like to express my gratitude to our collaborators, Judith Clements, Daniela Loessner, Jonathan Harris from Queensland University of Technology and Oliver Schilling from Universität Freiburg for sharing their data and exchanging ideas with us.

I would like to express my gratitude to the friends in the lab: Susanne Sölch who taught me several technologies; Dominik Alterauge for his help with qPCR experiments; Tom Schulze, Shuo Zhao, Thuy An Pham, Feng Yang, Xiaocong Geng and Yueyang Liu for their kind help in both lab work and life. There are still many other people I would like to thank: Julia Miller, Claudia Cerny, Tobias Dreyer and Johanna Felber. It is of great pleasure to meet and work with these people.

In the end, I would like to thank my family for their enormous support all these years. My father passed away last year due to stroke. I feel so sad and depressed all the time. Sometimes, I regret so much coming to Munich and wish I could spend more time with him. I really miss you.

Many thanks to all the people who helped me.

9 List of publications

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6. Ping Wang. OV-MZ-6 ovarian cancer cells overexpressing kallikrein-related peptidases 4, 5, 6 and 7: effects on expression of cancer-related genes. DAAD Workshop: German-Australian Network on Personalized Cancer Medicine. 2015, Brisbane, Australia. Oral presentation.

10 Appendix

10.1 Ovarian cancer FIGO stage system

Stage		Description
I		**Tumor confined to ovaries**
	IA	Tumor limited to 1 ovary, capsule intact, no tumor on surface, negative washings.
	IB	Tumor involves both ovaries otherwise like IA.
	IC	Tumor limited to 1 or both ovaries
	IC1	Surgical spill
	IC2	Capsule rupture before surgery or tumor on ovarian surface.
	IC3	Malignant cells in the ascites or peritoneal washings.
II		**Tumor involves 1 or both ovaries with pelvic extension (below the pelvic brim) or primary peritoneal cancer**
	IIA	Extension and/or implant on uterus and/or Fallopian tubes
	IIB	Extension to other pelvic intraperitoneal tissues
III		**Cancer found outside the pelvis or in the retroperitoneal lymph nodes, involves one or both ovaries**
	IIIA	Positive retroperitoneal lymph nodes and/or microscopic metastasis beyond the pelvis
	IIIA1	Positive retroperitoneal lymph nodes only
	IIIA1(i)	Metastasis $\leq$ 10 mm
	IIIA1(ii)	Metastasis > 10 mm
	IIIA2	Microscopic, extrapelvic (above the brim) peritoneal involvement ± positive retroperitoneal lymph nodes
	IIIB	Macroscopic, extrapelvic, peritoneal metastasis $\leq$ 2 cm ± positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen.
	IIIC	Macroscopic, extrapelvic, peritoneal metastasis > 2 cm ± positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen.
IV		**Distant metastasis excluding peritoneal metastasis**
	IVA	Pleural effusion with positive cytology
	IVB	Hepatic and/or splenic parenchymal metastasis, metastasis to extraabdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)

10.2 Ovarian cancer grading system

grade	description
GX:	The grade cannot be evaluated.
GB:	The tissue is considered borderline cancerous. This is commonly called low malignant potential (LMP).
G1	The tissue is well-differentiated (contains many healthy-looking cells).
G2	The tissue is moderately differentiated (more cells appear abnormal than healthy).
G3	Poorly differentiated cancer
G4	Undifferentiated

10.3 Primers used in the experiments

Gene		**5' - 3' Seq**	**UPL Probe**
HPRT1	L	TGACCTTGATTTATTTTGCATACC	73
	R	CGAGCAAGACGTTCAGTCCT	
KLK4	L	ATGGCCACAGCAGGAAAT	45
	R	CTACCAGAGACGAGCGATCC	
KLK5	L	AAGGCCCAACCAGCTCTACT	4
	R	CCGAGACGGACTCTGAAAAC	
KLK6	L	TGGTGCTGAGTCTGATTGCT	82
	R	CGCCATGCACCAACTTATT	
KLK7	L	CCTGCTCAGTGGCAATCA	86
	R	GGTGCACGGTGTACTCATTC	
BMP4	L	CTGCAACCGTTCAGAGGTC	17
	R	TGCTCGGGATGGCACTAC	
BMP5	L	AATCCAGCTCTCATCAGGACTC	47
	R	CAGGCTTGTTTTTGCTCACTT	
COL1A2	L	CTGGAGAGGCTGGTACTGCT	79
	R	AGCACCAAGAAGACCCTGAG	
COL5A2	L	ACAGGGTTTACAAGGACAGCA	79
	R	GGTCCAGGATCACCAGGTT	
KRT7	L	CAGGCTGAGATCGACAACATC	24
	R	CTTGGCACGAGCATCCTT	
KRT19	L	GCCACTACTACACGACCATCC	71
	R	CAAACTTGGTTCGGAAGTCAT	
MSN	L	AAGTTGCTCCCGCAGAGA	52
	R	TGTTCCTCATGCCACACCT	
JUNB	L	ATACACAGCTACGGGATACGG	49
	R	GCTCGGTTTCAGGAGTTTGT	
MMP1	L	GCTAACCTTTGATGCTATAACTACGA	7
	R	TTTGTGCGCATGTAGAATCTG	
F10	L	AGATTCAAGGTGAGGGTAGGG	15
	R	GACCACCTCCACCTCGTG	

10.4 Top 10 regulated genes in OV-KLK4-7 *vs.* OV-VC cells identified by the genome-wide RNA microarray analysis (D. Loessner, unpublished data)

Gene symbol	Description	Fold change
Top 10 upregulated		
CRIP1	cysteine-rich protein 1	184.07-323.46
KRT19	keratin 19	1.68-186.61
MSN	moesin	5.59-53.6
HES5	hairy and enhancer of split 5	6.31-52.93
JSRP1	junctional sarcoplasmic reticulum protein 1	14.57-34.79
UPK1B	uroplakin 1B	6.76-22.18
LRRC17	leucine rich repeat containing 17	3.67-18.35
HEY1	hairy/enhancer-of-split related with YRPW motif 1	3.39-16.39
PLCB2	phospholipase C, beta 2	2.25-14.83
PXMP4	peroxisomal membrane protein 4, 24kDa	2.46-14.15
Top 10 downregulated		
DLK1	delta-like 1 homolog	-29.52 to -47.83
MSX1	msh homeobox 1	-5.68 to -42.88
PRUNE2	prune homolog 2	-1.63 to -18.11
KRT7	keratin 7	-1.52 to -16.5
CADM1	cell adhesion molecule 1	-1.7 to -15.06
TOLLIP	toll interacting protein	-2.25 to -10.8
MAP1LC3B2	microtubule-associated protein 1 light chain 3 beta 2	-10.41 (only 1 probe)
ITM2A	integral membrane protein 2A	-3.45 to -10.09
ICA1	islet cell autoantigen 1.69kDa	-2.03 to -9.69
CDK14	cyclin-dependent kinase 14	-2.55 to -9.04
PRKACB	protein kinase, cAMP-dependent, catalytic, beta	-1.5 to -9.04

10.5 Significantly regulated proteins in KLK4-7-overexpression OV-MZ-6 ovarian cancer cells identified by proteomics analysis

Uniprot	p-value	Fc-value	Std. dev.	Name
P41222	0.000	4.1	0.8	Prostaglandin-H2 D-isomerase
Q7Z7G0	0.000	4.4	1.1	Target of Nesh-SH3
P33908	0.000	-2.4	0.4	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA
P26022	0.000	3.4	0.2	Pentraxin-related protein PTX3
O43405	0.000	3.7	1.1	Cochlin
P36955	0.000	3.6	0.3	Pigment epithelium-derived factor (PEDF)
P24592	0.001	3.1	1.1	Insulin-like growth factor-binding protein (IGFBP) 6
P05997	0.001	4.9	0.5	Collagen alpha-2(V) chain
P09341	0.001	2.5	1.6	Growth-regulated a protein (Gro-a, C-X-C motif chemokine 1, CXCL1)
P17936	0.001	2.7	1.3	Insulin-like growth factor-binding protein (IGFBP) 3
P07711	0.002	2.2	0.2	Cathepsin L1
P53634	0.002	2.2	0.3	Dipeptidyl peptidase 1
P01034	0.003	1.9	0.2	Cystatin-C
P10646	0.003	2.1	0.1	Tissue factor pathway inhibitor
Q9UBG0	0.004	-2.6	0.9	C-type mannose receptor 2
Q08629	0.004	2.1	0.5	Testican-1
P49746	0.004	2.4	0.9	Thrombospondin-3
P55268	0.004	2.7	0.8	Laminin subunit b-2
P49755	0.005	1.6	1.1	Transmembrane emp24 domain-containing protein 10
P13987	0.005	1.7	0.5	CD59 glycoprotein
O00300	0.009	-3.6	1.7	Osteoprotegerin
O94907	0.009	2.1	0.5	Dickkopf-related protein (DKK)-1
P01024	0.010	2.2	0.6	Complement C3
P32004	0.010	2.0	0.3	Neural cell adhesion molecule L1
Q9H3T2	0.013	-1.4	1.3	Semaphorin-6C
P12109	0.015	1.9	0.6	Collagen a-1(VI) chain
Q96EU7	0.015	2.1	2.4	C1GALT1-specific chaperone 1
Q99542	0.025	1.6	0.5	Matrix metalloproteinase-19
P09603	0.027	1.6	1.4	Macrophage colony-stimulating factor (MCSF)-1
O95407	0.033	1.5	0.4	Tumor necrosis factor receptor superfamily member 6B
Q9BTY2	0.038	1.5	0.5	Plasma alpha-L-fucosidase
P01137	0.041	1.6	0.5	Transforming growth factor (TGF)b-1
Q14563	0.042	1.7	0.2	Semaphorin-3A
P02786	0.043	1.9	0.1	Transferrin receptor protein 1

Uniprot ID and recommended name: according to uniprot database. Fc-value: fold change [173].

11 Abbreviations

2D	two dimensional
3D	three dimensional
Ab	antibody
BSA	albumin bovine serum
CA-125	serum Cancer Antigen 125
cDNA	complementary deoxyribonucleic acid
Ct	threshold cycle
DAB	3,3’-diaminobenzidine
DMEM	dulbecco’s modified eagle medium
DMSO	dimethylsulfoxide
DNA	desoxyriunicleic acid
dNTP	deoxyribonucleoside triphosphate
DPBS	dulbecco's phosphate-buffered saline
ECM	extracellular matrix
EDTA	ethylene diamine tretraccetic acid
EMT	epithelial to mesenchymal transition
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
h	hour
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HPRT	hypoxanthine-guaninephosphoribosyltransferase
IHC	immunohistochemistry
kDa	kilo Dalton
KLK	human tissue kallikrein-related peptidase
min	minute
MMP	matrix metalloproteinase
PAGE	polyacrylamide gel electrophoresis
PSA	prostate-specific antigen
PVDF	polyvinylidene difluoride
qPCR	quantity polymerase chain reaction
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
sec	second
TGFβ1	transforming growth factor beta 1
Triton	octylphenolpolyethylenglykol
TVUS	transvaginal ultrasound
UTR	untranslated region

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