Dissertation

Identification and characterisation of long non-coding RNA PANTR1 in clear-cell renal cell carcinoma

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

Dr. Maximilian Seles, e.h.

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Disclosures

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Abbreviations and Definitions (in alphabetical order)

ARSR: activated in RCC with sunitinib resistance
ASO: antisense oligonucleotides
AUC: area under the curve
BAP1: breast cancer-associated protein 1
bp: base pairs
CSS: cancer specific survival
ccRCC: clear-cell renal cell cancer/carcinomacDNA: complementary DNA
chRCC: chromophobe renal cell cancer/carcinoma
DFS: disease-free survival
DNA: deoxyribonucleic acid
EMA: European Medicines Agency
EMT: epithelial-to-mesenchymal transition
eSCC: oesophageal squamous cell cancer
EZH2: Enhancer of zeste homolog 2
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GEO: gene expression omnibus
H3K27: histone 3 lysine 27
HCC: hepatocellular carcinoma
HIF: Hypoxic-inducible Factor
HOTAIR: Homeobox transcript antisense RNA
lncRNAs: long non-coding ribonucleic acids
MALAT1: metastasis-associated lung adenocarcinoma transcript 1
miRNAs: micro RNAs
mRNA: messenger ribonucleic acid
NC: non-coding
NCBI: National Centre for Biotechnology information of the National Library of Medicine, USA
ncRNA: non-coding ribonucleic acid
NRF2-ARE: Nuclear factor erythroid 2-related factor 2 - antioxidant responsive element
nSCLC: non-small cell lung cancer
nSCC: nasopharyngeal squamous cell cancer
ORR: overall response rate

OS: overall survival
qRT-PCR: quantitative, real-time polymerase-chain reaction
PCR2: polycomb repressive complex 2
PDGF: Platelet-derived Growth Factor
piRNAs: piwi-interacting RNAs
polybromo1: PRBM1
POU3F3: POU class 3 homeobox 3
pRCC: papillary renal cell cancer/carcinoma
RKPM: reads per kilobase per million reads placed
RCC: renal cell cancer/carcinoma
RNA: ribonucleic acid
siRNAs: small interfering RNAs
SETD2: SET domain containing 2
snoRNAs: small nucleolar RNAs
snRNAs: small nuclear RNAs
SRLR: sorafenib resistance-associated lncRNA in RCC
TKI: Tyrosine kinase inhibitor
VEGF: Vascular endothelial Growth Factor
VEGFR: Vascular endothelial Growth Factor Receptor
VHL: von-Hippel Lindau

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Abstract

Non-coding RNAs (ncRNAs), notably long non-coding RNAs (lncRNA), have recently been under intensive investigation for their role in carcinogenesis. *POU3F3 adjacent non-coding transcript 1* (PANTR1) is a lncRNA with remarkable expression during foetal brain and renal development. Besides, it is known for a versatile role during carcinogenesis of different types of cancer. No available information exists about its role and a possible mode of action in clear-cell renal cell carcinoma (ccRCC).

In-silico data analysis of publicly available databases revealed PANTR1 expression to be confined to corresponding and healthy foetal and adult and cancerous human brain and kidney tissue. Moreover, it was significantly up-regulated in ccRCC compared to matched non-cancerous kidney tissue ($p<0.001$). PANTR1 expression was not associated with altered overall survival (OS) in these publicly available cohorts.

An siRNA-mediated knockdown approach in three different ccRCC cell lines demonstrated significant differences in cellular growth also by induction of apoptosis. We could further demonstrate significant differences in expression of angiogenic genes including the vascular endothelial growth factor-A (VEGF-A) by qRT-PCR. By use of publicly available datasets, VEGF-A and PANTR1 showed a significant correlation ($p=3.1x10^{-11}$, $R=0.28$, Spearman's rank correlation coefficient).
Using an *in vitro* angiogenesis assay, knockdown of PANTR1 in human umbilical vein endothelial cells (HUVECs) was established as a surrogate marker for angiogenesis. Several vascularisation patterns and endothelial cell migration were significantly reduced in PANTR1-knockdown cells compared to normal HUVECs.

LncRNA PANTR1 seems to be an oncogenic driver for ccRCC carcinogenesis with a promising potential as a future therapeutic target.

Zusammenfassung

Nicht kodierende, vornämlich lange nicht-kodierende RNAs, werden seit längerem intensiv hinsichtlich ihrer Rolle in der KKarzinomentstehung untersucht. *POU3F3-adjacent non-coding transcript 1* (PANTR1) ist eine lange, nicht kodierende RNA mit bemerkenswerter Expression während der fötalen Gehirn- und Nierenentwicklung. Sie ist aber auch für ihren vielfältigen Einfluss auf verschiedenen Grundpfeiler der Krebsentstehung bei unterschiedlichsten Karzinomen bekannt. Für das klarzellige Nierenzellkarzinom hingegen existieren noch keine dementsprechenden Daten.

Durch Datenanalyse öffentlich verfügbarer Sequenzierungsdatenbanken zeigte sich, dass die Expression von PANTR1 nur auf gesundes, und kanzerös verändertes Gehirn- und Nierengewebe (darunter auch der klarzellige Subtyp) beschränkt war. Die Expression von PANTR1 war darüber hinaus auch in Gewebeproben aus Tumoren des klarzelligen Subtyps im Vergleich zu benachbartem gesunden Nierengewebe signifikant erhöht. Die Expression von PANTR1 war nicht mit unterschiedlichem Gesamtüberleben bei Patient*Innen mit klarzelligem Nierenzellkarzinom assoziiert.

In drei verschiedenen klarzelligen Nierenzellkarzinomzelllinien wurden nach erfolgreicher siRNA - mediierter Reduktion der Expression von PANTR1 signifikante Unterschiede in der Wachstums- und der Apoptoserate der Zellen gefunden. Mittels qRT-PCR konnten signifikante Unterschiede in der Expression von Angiogenesegenen (z.B. VEGF-A) gefunden werden. Eine Korrelationsanalyse zwischen VEGF-A und PANTR1 zeigte signifikante Resultate ($p=3.1x10^{-1)}$, R=0.28, Spearmans Korrelationskoeffizient).
Ein *in-vitro* Angiogenese-Assay in humanen endothelialen Nabelschnurzellen (HUVEC) konnte erfolgreich etabliert werden, um die Angiogenese als wichtigsten Schlüsselfaktor beim klarzelligen Nierenzellkarzinom besser beleuchten zu können. Migrations- und Vaskularisationsparameter waren hier nach Knockdown von PANTR1 signifikant reduziert.

Die bereits für andere Karzinome etablierte Hypothese der onkogenen Funktion von PANTR1 scheint für das klarzellige Nierenzellkarzinom bestätigt worden zu sein.

1 Introduction

As preparation for this thesis, an open-access full-text review on the role of lncRNAs in renal cell carcinoma was published as:

Seles, M.; Hutterer, G.C.; Kiesslich, T.; Pummer, K.; Berindan-Neagoe, I.; Perakis, S.; Schwarzenbacher, D.; Stotz, M.; Gerger, A.; Pichler, M. Current Insights into Long Non-Coding RNAs in Renal Cell Carcinoma. International Journal of Molecular Sciences. 2016, 17, 573 [2].

In 2016, it was the first publication ever to give a comprehensive overview on this topic. This introduction will reflect parts this review and updated information as of 2016 will provide a comprehensive and revised overview on lncRNA and RCC.

As lncRNA PANTR1 was not a known player at that time, an extra section is dedicated to several aspects regarding this specific lncRNA and its genetic background concerning the protein-coding neighbour gene POU3F3.

1.1 Renal Cell Carcinoma

1.1.1 Epidemiology and aetiology

Renal cell carcinoma (RCC) accounts for 3% of all newly diagnosed (with an increase since 1983) and 2% of all cancer deaths (with a decrease since 1983) in Austria in 2017 [5,6]. It represents the 3rd most common urological malignancy with an incidence rate of approximately 99.200 new RCC cases and 39.100 RCC-related deaths in the European Union in 2018 [7,8].

RCC is not common in children and represents under 10% of all renal tumours [9].

We currently observe a 5-year OS rate of 49% in adults when accounting for all subtypes of RCC. This rate has improved since 2006 most probably due to the increased incidental detection of RCC. The introduction and the subsequent widespread use of computed tomography and magnetic resonance imaging as well as the introduction of useful systemic therapies have led to a decrease of death rates during these last 20 years. Unfortunately, death rates continue to rise in the elderly [10–12]. This seems to be a consequence of age, which represents the most relevant risk factor for RCC in our continuously ageing population in the western world.

The most relevant risk factors are age, first-degree-relatives with RCC, diabetes, obesity, smoking and hypertension. Reduction of body weight in case of obesity, physical activity, cessation of tobacco smoking und the consumptions of moderate amounts of alcohol are regarded as protective factors.
In general, when regarding almost all subtypes of non-hereditary RCC, therapeutic options include active surveillance (in case when any active therapy is not suitable), surgical partial and radical nephrectomy with or without lymphadenectomy and systemic medical therapy [8,13,14].
Unfortunately, up to one third of all RCC patients will present with primary metastatic disease, whereby conventional systemic chemo- and/or radiotherapy shows very moderate to no benefit at all. Currently, systemic therapeutic options include metastasectomy and systemic medical options with tyrosine kinase inhibitors (TKIs) and/or immunotherapy [8].

1.1.2 Subtypes and molecular characteristics

90% of all solid lesions of the kidney are classified as RCC [8]. For systematisation purposes, RCC is classified into several subtypes. Currently, they are organized according to the *WHO 2016 classification of Tumours of the Urinary system and the Genitals* [15].
The three most common subtypes, which account for almost 98% of all tumours, are:

- ccRCC,
- papillary RCC (pRCC) and
- chromophobe RCC (chRCC).

All other subtypes occur very rarely and will not be discussed.

1.1.2.1 Clear-cell renal cell carcinoma

CcRCC represents the most common subtype with approximately 70 to 80% of all RCC cases [9].

90% of all sporadic cases of ccRCC develop along the same, clearly defined pathway. Presumably decades ago, a mutation in one of the two alleles of the *von-Hippel-Lindau* (VHL) gene on chromosome 3p occurs. VHL gene encodes for the VHL protein, which acts as the guardian of the *hypoxic-inducible factor* (HIF). HIF consists of 3 subunits: HIF-1α, HIF-2α und HIF-1β. While HIF-1β is ubiquitously expressed within the cell, HIF-1α und HIF-2α act as a pair constantly trying to dimerize with HIF-1β. VHL protein is the oxygen-sensing subunit of a E3-Ubiquitin-ligase complex that inactivates the “HIF-1α - HIF-2α pair” under normoxic conditions. Consequently, ubiquitination leads to proteasome-mediated degradation.
Under hypoxic conditions, VHL does not ubiquitinate the HIF-1α subunits, which leads to activation of the entire HIF complex. This activates hypoxia-response genes, among them Vascular Endothelial Growth Factor-A (VEGF-A), Platelet-derived Growth Factor (PDGF), Erythropoietin and several others.
In ccRCC carcinogenesis, a subsequent second event is needed to finally initiate the formation process. Mostly, a mutation on the 2nd VHL allele on somatic chromosome 3p occurs. This leads to inadequate deactivation of VHL activating HIF and all its downstream targets [16–21].

Deletion of chromosome 3p also leads to several other key events during ccRCC carcinogenesis: Inadequate expression of the *chromatin remodelling genes SET domain containing 2* (SETD2), *polybromo 1* (PBRM1) and *breast cancer-associated protein 1* (BAP1). While BAP1 is part of the polycomb repressive complex (PCR), SETD2 there influences a methyltransferase and PBRM1 is part of the SWI/SNF complex. These three genes are regarded as altered in ccRCC in addition to VHL which lead to alterations in chromatin structure, abnormal DNA transcription and altered DNA damage repair [16,17].

Current therapeutics mostly aim for downstream targets of the VHL-HIF - axis including the PDGF-Receptor, MET, VEGF and VEGF-Receptors 1, 2 and 3 [21].
Several substances are available on the market (see Table 1).

Table 1: Table listing all available and EMA-licensed tyrosine kinase inhibitors and their targets for the treatment for mRCC [8,20]

Name	**Inhibited tyrosine kinase**
Sunitinib	VEGFR-1, −2 (KDR) and−3 PDGFR-α and β c-KIT FLT-3 CSF-1R RET
Sorafenib	VEGFR-1, −2 and−3 PDGFR-β FLT-3 c-KIT RET RAF
Pazopanib	VEGFR-1, −2, and−3 PDGFR c-KIT
Axitinib	VEGFR-1, −2, and−3
Lenvatinib	VEGFR-1, −2, and−3 PDGFR-α Ret KIT FGFR 1to FGFR4
Cabozantinib	VEGFR-1, −2, and−3 MET AXL
Tivozanib	VEGFR-1, −2, and−3

Downstream to several of these tyrosine kinases, other growth factors influence the *mammalian target of rapamycin* complex (mTOR). This mTOR complex is composed of two subunits, mTORC1 and mTORC2. It is activated via the *phosphatidyl-inositol 3*/PI3-Kinase and *Tuberosis sclerosis*/TSC - complex. mTORC1 is inhibited by substances from the class of mTOR inhibitors, in case of mRCC these are Everolimus and Temsirolimus. Both substances were frequently used from 2000 to 2015, but have now been resettled to last-line therapies in mRCC [8,22]. A monoclonal antibody against VEGF (Bevacizumab) was also frequently used during this period, but now also has been replaced in most parts of the world [8,13,14].

In 2021, systemic therapeutic options for ccRCC include mostly a combination of immune checkpoint inhibitors against CTLA-4 or as PD-1/Receptor ligand (Pembrolizumab, Ipilimumab, Nivolumab) and single- and/or multikinase-inhibitors as explained above (see Table 1).

1.1.2.2 Papillary RCC

pRCC is the 2nd most common subtype of RCC which accounts for approximately 10 to 15% of all cases. In up to 50% of cases, it presents simultaneously with multiple tumours at different local uni- and bilaterally. It is divided into two subgroups: pRCC type I and type II which, from a biological and clinical point of view, differ greatly.

Patients with pRCC type I usually profit from a better prognosis compared to pRCC type II. They often present as pseudocystic tumours leading to radiological undergrading/-staging as Bosniak IIF or Bosniak III cysts [8].
Type I tumours are not associated with mutated *VHL* function. They are rather hypovascularised tumours and are associated with mutations of the proto-oncogene c-MET [8].
Patients with type II tumours usually present at higher stage and grade with a higher risk of primary metastatic disease. Usually, they suffer from a lower 5-year cancer specific survival (CSS) [23,24]. PRCC type II can correspond to the familial “Hereditary Leiomyomatosis Renal Cell Cancer” syndrome and needs early surgical intervention. It is characterized by a defect of the *fumarate hydratase*, SETD2 mutations and activation of the *Nuclear factor erythroid 2-related factor 2 - antioxidant responsive* element (NRF2-ARE) pathway but pseudo-hypoxic effects associated with overexpressed HIF are also reported [18,25].

1.1.2.3 Chromophobe RCC

Chromophobe RCC accounts for 5% of subtypes of RCC [8]. Genetic investigations found *Phosphatase and tensin homolog*/PTEN and *tumour protein 53*/TP53 to be altered in a relevant number of chRCC patients [26]. Regarding familial genetic RCC syndromes, chRCC is associated with the Birt-Hogg-Dubé-Syndrome which exhibits mutations in the *folliculin* gene. However, sporadic forms also exhibit multiple copy number alterations in chromosomes 1, 2, 6, 10, 13 and 17 [27,28].

1.2 Long non-coding RNAs

Different kinds of RNAs exist in eukaryotic human cells. Most easily, they are classified into coding and non-coding/untranslated RNAs whereupon messenger RNA (mRNA) is *the* coding RNA and therefore represents the “blueprint” for proteins. NcRNAs outnumber mRNAs by far and can furtherly be classified into housekeeping RNA (transfer RNA (tRNA) and ribosomal RNA (rRNA)) and regulatory RNAs. Regulatory RNAs are then divided into two subgroups at an arbitrary cut-off at 200 nucleotides: short and long non-coding RNAs.

All ncRNAs display comparable features regardless of their length: their 7-methylguanosine triphosphate cap at the 5’ and their poly-adenylated tail at the 3’ end, their high conservation across different species, their translation status depending on histone and chromatin phase and the (mostly) transcription by RNA polymerase II [2].
The class of short ncRNAs comprises several different subtypes: micro RNAs (miRNAs), piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), circular RNA (circRNA), small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs) with probably more to be discovered. They might also undergo re-classification due to widespread ongoing research [29,30].

All lncRNAs share one feature: their length of more than 200 nucleotides. However, they are a diverse group of molecules with very few common structural or functional characteristics apart from their non-coding potential.
Nevertheless, their genomic location and their mode of action are commonly classified as follows. Their location in relation to protein-coding neighbour genes can be described as intergenic, bidirectional, intronic, sense as well as antisense [31]. Their function may be

location-specific but they can function in cis (at their locus) or in trans (at any other locus) within the genome [32,33].
LncRNAs can exert RNA- and protein related functions due to their 3D structure based on the Watson-Crick-principle. Their expression is tissue-specific and correlates with overall mRNA expression. They can be located in the nucleus and in the cytoplasm and can exert different functions depending on their localisation [31].

They can act as a [31,34]

1. protein recruiter and consequently enhancer of gene expression,
2. scaffold to host 2 or more different proteins,
3. direct regulator of transcription to repress gene expression,
4. decoy to prevent transcription,
5. competitor RNA to e.g. sponge miRNAs or to dock to mRNA to prevent transcription,
6. recruiter of double stranded RNAs to promote mRNA degradation and
7. promoter of telemerase elongation.

1.2.1.1 Long intergenic non-coding RNA (LincRNA)

LincRNAs are a sub-class of lncRNAs and represent about 50% of all lncRNA. They are of the same length as lncRNAs but do not overlap with annotated protein-coding genes. Currently, GENCODE describes 13.255 lincRNA transcripts arising from 8.598 genes in ccRCC. There are not as well conserved as other lncRNAs and exhibit less conservation among species compared to protein coding genes [35].

1.2.2 Long non-coding RNAs in RCC

In mostly studies base on microarrays and polymerase-chain reaction (PCR), several thousand (number differs greatly between the publications) aberrantly expressed lncRNA were identified out of an overall amount of approximately 35.000 lncRNAs [36–40].

The number of up and down-regulated lncRNAs in all the different publications shows great variance [36,38,39,41] which makes it difficult to draw a final conclusion.

In almost all publications, the authors tried to link their findings to clinical data, but there is not a single common lncRNA that could be defined as the prime target for further research. This might be caused by the heterogenous biology of RCC in general but also by the great intra- und intertumoral heterogenicity in RCC.

1.2.2.1 Specific lncRNAs in RCC

For other types of cancer as well as for many physiological reactions famous lncRNAs have been described. The role of some famous lncRNAs with regard to their role in RCC will be reflected as an overview in the following section.

HOTAIR

Homeobox transcript antisense RNA (HOTAIR) is a prime epigenetic regulator of transcription and interacts with the *Enhancer of zeste homolog 2* (EZH2), a subunit of the PCR, which leads to *histone 3 lysine 27* (H3K27) trimethylation and thus gene repression [42,43]. It is transcribed from the antisense strand of the HoxC gene on chromosome 12 and it was the first lncRNA whose role in cancer was discovered [44]. HOTAIR is overexpressed in different types of cancer [44–49].
HOTAIR expression is elevated in RCC cells compared to normal renal tissue. SiRNA-mediated knock-down of HOTAIR results in reduced migration and proliferation [50,51].

MALAT1

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT 1), also known as NEAT2, is a ubiquitously expressed lncRNA, which shows high conservation across certain species [52]. MALAT1 was recently identified as an important oncogene in several types of cancer. Expression of MALAT1 is higher in cancerous tissue compared to normal tissue. It promotes migration, invasion, cell proliferation, apoptosis and the development of metastasis [52–57]. In ccRCC, MALAT1 is also overexpressed compared to non-cancerous adjacent renal tissue samples [58–60]. A knock-down of MALAT1 in RCC cell lines showed decreased cell proliferation rate, decreased migration but increased invasion and apoptosis rates [58–60].

Direct stimulation of MALAT1 by c-fos was demonstrated in ccRCC. C-fos is a transcription factor conjointly activated with c-jun in the downstream pathways of VHL [59,60].

Furthermore, MALAT1 seems to act as an epigenetic regulator. Correlation between MALAT1 and EZH2 could be demonstrated. After inactivation of MALAT1, decreased expression of EZH2, Beta-Catenin, H3K27me3 and c-myc could be shown. E-Cadherin represents a tumour suppressor gene typically down-regulated in EMT in RCC [61]. Beta-Catenin participates in the Wnt/Beta-Catenin pathway which is activated by EZH2. This activates Wnt target genes, such as c-myc to drive carcinogenesis [62,63].

A reciprocal effect between MALAT1 and miRNA-205, a tumour suppressor in RCC, was demonstrated [59]. MALAT1 regulates *Zinc finger E-box binding homeobox 2*/ZEB2 expression via sponging of miR-200 in a dose-dependent manner and consequently influences EMT [60]. All these results show possible pathways of MALAT1 to induce carcinogenesis by facilitating EMT, cancer progression and metastasis.

MALAT1 also seems to be of interest from a biomarker point of view. High levels of MALAT1 correlate significantly with pathologic T-stage, tumour diameter and the presence of lymph node metastases. Furthermore, MALAT1 expression could stratify an OS in even multivariate analysis in ccRCC [58].

H19

H19 represents a maternally imprinted lncRNA, which is exclusively expressed during the embryonic period. It is then fully repressed in most human tissues after birth but can be reactivated during carcinogenesis [64–66]. H19 also plays a key role in mesenchymal-to-epithelial transition by influencing the function of EZH2, Beta-Catenin and E-Cadherin [64].

H19 acts as an oncogene in several types of cancer, including breast, bladder, ovarian and gastric cancer, as well as glioma [67–69]. In analogy to other types of cancer, H19 is overexpressed in ccRCC. Knock-down of H19 results in decreased migration, invasion and cellular growth [70].

When we refer to lncRNAs as a biomarker, H19 is a favourable candidate. Significant correlation with pathological tumour stage and lymph node as well as distant metastases could be demonstrated. Furthermore, higher expression of H19 is associated with shorter OS [70].

HIF1A Transcripts

Two lncRNAs at the 3' and 5' end antisense to HIF-1α, named 5' HIF-1α and 3' HIF-1α are expressed at high and intermediate levels in non-papillary RCC and 3' HIF-1α is expressed at low levels in pRCC. No information about a correlation to clinical data is yet available [71,72].

Other lncRNA

An almost countless number of other lncRNAs have been discovered during the last two decades. Table 2 lists a few of them to display the variety of functions in RCC. It seems not useful to compile a complete list as investigations on new lncRNAs will appear every week from now and this list will be outdated as soon as it is be published.

Table 2: List of many of the currently known LncRNAs with described function in RCC

Name	**Suppressor/ Oncogene**	**Function**	**Effect**
NBAT1 [73]	Suppressor	Regulator of cell proliferation and neuronal differentiation	Decreased cell proliferation, migration and invasion

RCCRT1 [74]	Oncogene	unknown	Increased cell migration and invasion
CADM1-AS1 [75]	Suppressor	Unknown, CADM1 = membrane protein involved in cell-to-cell interactions	Decreases apoptosis, migration and growth rates
SPRY4-IT1 [76]	Oncogene	Unknown, SPRY4=inhibitor of the receptor-transduced mitogen-activated protein kinase (MAPK)	Stimulation of cell migration, proliferation and invasion
DUXAP8[77]	Oncogene	unknown	Stimulation of proliferation & invasion
TUG1 [78]	Suppressor	unknown	Inhibition of proliferation, migration, invasion. stimulator of apoptosis
Z38 [79]	Oncogene	unknown	Stimulation of proliferation, migration, invasion, MET.
EGOT[80]	Suppressor	unknown	Inhibition of proliferation, migration and invasion. Induction of apoptosis
HEIRCC [81]	Oncogene	unknown	Stimulation of cell proliferation and apoptosis, cell migration, invasion and MET.
DHRS4-AS1 [82]	Suppressor	unknown	Inhibition of proliferation, invasion, cell cycle progression. Stimulation of apoptosis.

FILNC1 [83]	Suppressor	Decrease of glucose uptake through c-myc upregulation	Increases c-myc levels via AUF-1
THOR [84]	Oncogene	unknown	Stimulation of cell growth, viability, proliferation. Induction IGF2, GLI1 and Myc expression.
Linc00997[85]	Oncogene	Regulation of S100A11	Reduction of migration in ccRCC
Linc00037 [86]	Oncogene	Binding to EGFR	Stimulation of proliferation, inhibition of apoptosis
MFI2-AS1 [87]	Oncogene	Unknown	Promotion of metastatic spread
ROR [88,89]	Oncogene	Stimulation of MiRNA206/VEGF axis	Stimulation of proliferation, reduction of apoptosis, p53 expression, increase of c-myc
BX357664 [90]	Oncogene	Inhibition of TGF-β1/p38/HSP27 signalling	Stimulation of migration, invasion, proliferation.
ENSG00000241684 [91]	Suppressor	Unknown	impaired cell proliferation and reduced invasion
DANCR [92]	Suppressor	Unknown	Inhibition of proliferation, migration and invasion. Promotor of apoptosis.

1.2.2.1 LncRNAs as biomarkers in RCC

From a technical point of view, detection of lncRNAs from blood samples (“liquid biopsy”) is feasible. Due to the smaller difference in expression compared to the background noise, the process is more challenging [93]. However, a panel of five different lncRNAs (PVT1, linc00963, PANDAR, lncRNA-LET and PTENP1) was able to differentiate between ccRCC, benign renal tumour and healthy individuals with an area under the curve (AUC) of 0.8 (validation set) with a sensitivity and specificity between 80 and 90% depending on the sample set [94]. For pRCC, a 17-lncRNA and for ccRCC, a 11-lncRNA panel was created based on TCGA data with an AUC of 0.91 and 0.83, respectively with no validation yet [95,96].

By now, four different studies with a total of 1777 included patient samples have investigated different lncRNA panels to improve prediction of ccRCC. All followed the same principle of choosing one or several lncRNAs and to stratify patients along the selected lncRNAs.

1.2.3 LncRNAs and therapy in RCC

1.2.3.1 LncRNA-directed therapeutics in cancer

LncRNAs have been proposed to serve as target for directed therapies. Despite the long-lasting research, no single clinical trial is currently registered under http://www.clinicaltrial.gov.

Several concepts for lncRNA-directed therapy exist. They include siRNAs, antisense oligonucleotides (ASO), aptamers and substances that regulate the environment of lncRNA expression.

SiRNAs and ASO rely on the same principle of direct blockage of lncRNAs. While siRNAs work in cytoplasm only, ASO function regardless of their target location.
Though, ASO can encounter difficulties in interaction with a specific lncRNA as they undergo secondary restructuring. Aptamers are also oligonucleotides, but bind to secondary structures to bypass this inconvenience [97].

Recently, a completely different strategy has been applied in bladder cancer research: Therapeutic substance BC-819 influences the promoter region of lncRNA H-19 and has shown significant effect on bladder cancer [68,98–100].

Not a single study focusing on lncRNA-directed therapy in RCC has yet been reported.

1.2.3.2 LncRNAs and resistance to therapy

Several lncRNAs might be associated with resistance mechanisms to current therapies in RCC. Most of the studies have been performed in settings when the treatment was conducted with a tyrosine kinase inhibitor (Sunitinib or Sorafenib).
Regarding Sunitinib, a new lncRNA named *"activated in RCC with sunitinib resistance"* (ARSR) was found to interact with miR-449 and miR-34 which increases the levels of AXL and c-MET which seems to lead to resistance [101].
Regarding Sorafenib, a the newly discovered lncRNA *"sorafenib resistance-associated lncRNA in RCC"* (SRLR) interacts with NF-KB, interleukin-6 and *Signal transducer and activator of transcription/*STAT3 pathway and which influences its targets VEGFR and PDGFR [102].

Six years ago, PD/PD1-Ligand inhibitors where introduced to the market with currently two different substances available for RCC patients: Nivolumab (also in combination with CTLA4 inhibitor Ipilimumab) and Pembrolizumab. Several lncRNAs have been proposed to influence this mode of therapy, among them MALAT1 and HOTTIP [103].

1.2.4 Concluding remarks

LncRNA research in RCC has introduced some promising candidates for further investigation, but no breakthroughs have been published yet. Neither have lncRNAs achieved to position themselves as potential biomarkers in any setting during the treatment of RCC. Further research is warranted and needed to elucidate a possible major role for lncRNAs in RCC carcinogenesis and therapy.

1.3 Long intergenic non-coding RNA PANTR1

LincRNA *POU3F3 adjacent non-coding transcript 1* (PANTR1, LncRNA 1158, Linc01158, Linc-POU3F3 or LincBRN1a) is a non-protein coding transcript with four to ten (depending on the source) variants about 4-kb upstream of the *POU class 3 homeobox 3 (*POU3F3) gene on chromosome 2q12.1 [104]. Protein-coding neighbour gene POU3F3 is a well-known key player during the foetal development of the human central nervous system and the kidneys.

1.3.1 POU3F3

This section is dedicated to the POU3F3 gene as its expression during foetal development, its function as well as its neighbour location in regard to PANTR1 are of interest.

1.3.1.1 General

*POU class 3 homeobox 3 (*POU3F3, Gene ID: 5455) is a protein-coding gene with strong conservation across different species (e.g. mouse, rat, zebrafish, frog and Homo sapiens). It resides on chromosome 2q12.1 on the forward strand [105]. It displays several functions, which mostly occur during the foetal development in humans. It is responsible for correct development of parts of the central nervous system [106], the kidneys [107] and the maxillary bone [108]. Several other functions as a regulator of transcription by e.g. influencing RNA polymerase II have been reported [109].

1.3.1.2 Wild-type mutations in humans

Humans with mutated POU3F3 gene display with a wide range of functional and morphological changes. They range from mild cognitive impairment and slower motor development [106] to severe impairment with facial disorders and cerebral atrophies [110]. Surprisingly, no impairment of kidney functions was observed in humans. Only in rodents, data for POU3F3-deficient individuals exist. In POU3F3 knockout mice models, life was not possible due to neonatal renal failure [107]. Though, this does not exclude the possibility of POU3F3-associated renal failure in humans as they simply could not have been investigated right after neonatal death regarding this one mutation.

1.3.1.3 Kidneys

POU3F3 is responsible for the correct formation of the distal convoluted tube, the macula densa, and Henle's loop during mammalian development. In adult kidneys, POU3F3 was found only in the thick ascending loop of Henle [111]. Missense mutation also leads to a decreased number of nephrons. Though, no histological or ultrastructural lesions of Henle's loop cells or glomerular cells could be observed in homozygous mutant mice [111].

1.3.1.4 Central nervous system

In 1992, first discoveries on the function of POU3F3 in the human brain could be achieved [112]. POU3F3 seems to be responsible for the correct layering of neurons in layers II and IV in the neocortex [113–115] and for the correct development of the auditory epithelium [116]. Furthermore, POU3F3 does not seem to be responsible for morphological changes only, but also appears to be a master regulator of post-traumatic stress disorder [117].

1.3.1.5 Tumours

There are discrete and yet unverified hints that dysregulation of POU3F3 expression may lead to development of lymphomas [118] and conjunctival melanomas [119] in humans as well as breast cancer in rats [120].

1.3.2 PANTR1

1.3.2.1 General

No publication or review on PANTR1 exists, so all of this general information is gathered mostly from recognized and highly reliable online resources as NCBI gene database (accessible under https://www.ncbi.nlm.nih.gov/gene; [121] or the Ensembl Project (accessible under http://www.ensembl.org)[122]).

PANTR1 (official symbol) also resides on chromosome 2q12.1 on the reverse strand in direct proximity to the POU3F3 gene with no overlapping sequences (see Figure 1).

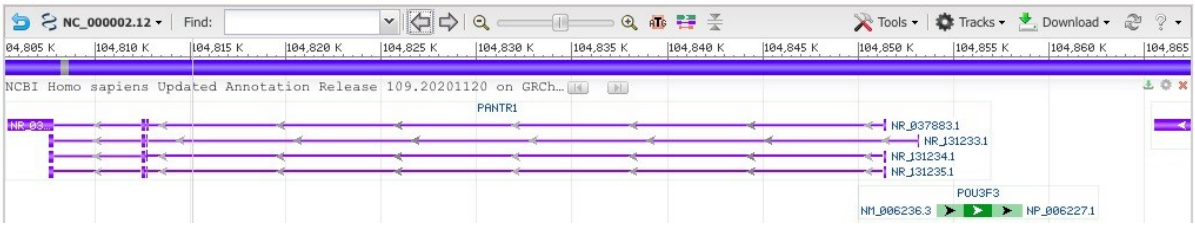


Figure 1. Screenshot from the NCBI gene database showing the direct proximity of POU3F3 gene (in green) and PANTR1 gene (all 4 transcript variants, in purple) with no overlapping sequence. From [122].

NCBI gene database reports four splice variants (see Figure 1) but Ensembl reports 10 different splice variants – none of them with protein coding function (see Table 3). Length of those transcripts spans from 607 to 5286 base pairs. No information on different functions or malfunctions of certain transcripts is yet available. Its function in normal tissues (apart from cancerous tissues) remains mostly uncertain. No knockout-models e.g. in rodents have been reported yet.

Thera are hints that PANTR1 positively influences POU3F3 mRNA expression [123].

Table 3: Table of all transcript variants of PANTR1 from Ensembl Consortium database. Data based on GENCODE data, length in base pairs. Modified after [122].

Name	Transcript ID	Length (in base pairs)
PANTR1-208	ENST00000661120.1	5286
PANTR1-207	ENST00000659645.1	2650
PANTR1-206	ENST00000653760.1	1725
PANTR1-210	ENST00000665955.1	1598
PANTR1-209	ENST00000664478.1	776
PANTR1-205	ENST00000458253.5	747
PANTR1-204	ENST00000454729.1	722
PANTR1-202	ENST00000443988.5	675
PANTR1-201	ENST00000413121.5	637
PANTR1-203	ENST00000447876.5	607

1.3.2.2 Role of PANTR1 in other types of cancer

No information on the role of PANTR1 in any subtype of RCC had existed until this dissertation had been started and until the publication resulting from it was published [1]. However, a lot of information on its versatile role in numerous types of cancer has been published (for details, please see Table 4).

Considering all publications available, PANTR1 always remains an oncogene with mostly the same pattern of influence on the different cancer types. It is always upregulated in cancerous compared to normal tissue.

Table 4: Table listing all publications on PANTR1 in different types of cancer. HCC=Hepatocellular carcinoma, eSCC=oesophageal squamous cell cancer, nSCC=nasopharyngeal squamous cell cancer, nSCLC=non-small cell lung cancer, ccRCC=clear-cell renal cell cancer.

Cancer type	**Publication(s)**	**Up-/Downregulation** ↓ ↑	**Further information**	**Clinical correlation**
HCC	[124]	proliferation ↑ migration ↑ invasion ↑	Inverse correlation with POU3F3 mRNA	yes
Glioma	[125,126]	Proliferation ↑ viability ↑ angiogenesis ↑	- Inverse correlation with POU3F3 mRNA - consecutive upregulation of FGF, bFGFR, VEGF-A, and Angio	yes
eSCC	[123,127–129]	Stroma tissue ↑ DNA methylation ↑ colony formation ↑ proliferation ↑	Regulates cisplatin resistance, induces cancer-associated fibroblasts, PANTR1 packed in exosomes for transfer, induces POU3F3 mRNA expression	yes
Breast Cancer	[130]	apoptosis↓ proliferation ↑	Inactivation of caspase 9	yes
Prostate Cancer	[131]	not known	Microarray study	no
Gastric Cancer	[132]	proliferation ↑	Influence of distribution of regulatory T cells, Recruitment of TGF-beta which increased the phosphorylation of SMAD2/3	yes
nSCC	[133]	Invasion ↑ migration ↑	Upregulation of TGF-1 Beta	yes
Cervical Cancer	[134]	proliferation ↑ invasion ↑	PANTR1 activated by SP-1, targets miRNA127-5 und thus FOXD1	yes

Colorectal Cancer	[135]	apoptosis↓ MET ↑ invasion ↑	autophagy↑, influences BMP signalling	yes
nSCLC	[136]	proliferation ↑ invasion ↑ migration ↑	Produces suppression of miRNA30d-5p	no
ccRCC	[1]	Proliferation ↑ angiogenesis ↑ apoptosis↓ migration ↑	Stratification for DFS and OS	yes

1.3.2.3 Role of PANTR1 in non-cancerous conditions

PANTR1 also plays a role in non-cancerous conditions. It was found to be overexpressed in cardiac in-stent restenosis patients. There, it also induces phenotypic expression of vascular smooth muscle cells via the PANTR1/ miR-449a / KLF4 signalling pathway [137].

Other non-cancerous conditions have not been investigated yet.

Hypothesis and Aim

In humans, PANTR1 is mainly expressed in brain and kidney tissue with emphasis on the embryonic period. Furthermore, PANTR1 has been proposed as relevant oncogene in different kinds of cancer, among them glioma. Glioma is highly dependent on angiogenesis. The same effect is known and even used for therapy in ccRCC. PANTR1 has never been investigated in any subtype of RCC including ccRCC.

Therefore, the aims of this dissertation were to

- identify whether PANTR1 plays a significant role in ccRCC and
- to dissect its different levels of influence on a cellular level with regard to the famous *hallmarks of cancer* [138,139].

Future Perspectives

Further studies can be based on these findings to decipher the underlining mode of action and possible interaction on the protein-coding and non-coding level of ccRCC.

Furthermore, PANTR1 could be identified in further cancer entities (among them also other subtypes of RCC).

PANTR1 alone or in combination with others could be identified as a biomarker in the preoperative, postoperative and metastatic setting with or without therapy.

2 Materials and Methods

2.1 Analysis of PANTR1 RNA expression and calculation of survival data

RNA sequencing data for PANTR1 in different normal adult human tissue types was obtained from the publicly available NCBI Entrez gene database (PMID=24309898) (accessible under https://www.ncbi.nlm.nih.gov/gene/?term=ENSG00000233639) There, PANTR1 RNA expression profiles from 95 different human individuals with 27 different tissue types are hosted which were originally created by Fagerberg *et al.* [140].

RNA sequencing data for PANTR1 during foetal development was retrieved from the same source (PMID=26076956) based on a publication of Szabo *et al.*[141,142].

RNA sequencing data for PANTR1 and its mRNA expression profile in different types of cancer were obtained from the publicly available GEPIA Server (accessible under http://gepia.cancer-pku.cn/detail.php?gene=LINC01158 [143,144]. They merge “The cancer genome atlas/TCGA” and the “Genotype-Tissue Expression/GTex” projects data to create a more comprehensive gene database. The retrieved figure was graphically modified for clarification and illustration purposes.

Disease-free (DFS) and overall survival (OS) data of patients suffering from ccRCC, chRCC and pRCC were obtained from the same source [143,144]. Patients were stratified according their PANTR1 expression at a cut-off of 50%. DFS and OS were calculated with Gepia’s integrated calculator (accessible under http://gepia.cancer-pku.cn/detail.php?clicktag=correlation###) because the data is not to be extracted from this database.

Data from NCBI’s gene expression omnibus (GEO) database (access number GSE53757, accessible under https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53757) were retrieved. Regarding ccRCC, it contains 144 human samples matching normal (n=72) vs. ccRCC tissue (n=72) with RNA expression data of PANTR1 which originates from a publication by von Roemeling *et al.* [145]. The data was tested with Kolmogorov-Smirnoff’s and Shapiro Wilk’s test which resulted in a non-parametric distribution. A Mann-Whitney-U signed rank test for 2 non-paired, non-parametric samples was performed and the result was illustrated in a boxplot.

2.2 Cell culture and applications

2.2.1 Cell culture

To diminish a possible selection bias, three different ccRCC cell lines were used.

Human ccRCC cell lines RCC-FG1/KTCTL-26 and RCC-MF/KTCTL-1M were obtained from CLS (Cell Lines Service; Eppelheim; Germany). Cell line RCC 769-P was obtained from ATCC (American Type Culture Collection; Manassas, VI; USA). All three cell lines were cultured in RPMI1640 growth media (Gibco-ThermoFischer Scientific; Vienna; Austria) which contains, amongst others, 10% foetal bovine serum (Hyclone-GE Healthcare; Frankfurt am Main; Germany) and a 1% penicillin/streptomycin mixture (Gibco-ThermoFischer Scientific; Vienna; Austria).

Human umbilical vein endothelial cells (HUVECs, owned by our own lab) were cultured in EBM™-2-Medium (EGM™-2 SingleQuots; Lonza; Basel; Switzerland) with the necessary supplements.

All cell lines were kept at 37°C in a humidified 5% CO_2 atmosphere. For each experiment, the pre-evaluated optimal cell number was cultivated in a suitable well format. Cells were counted by using an automated cell counter (TC-20, Biorad; Berkley; CA; USA) and then used.

2.2.2 Transient transfection with siRNAs

All three cell lines were transfected the same way with two different siRNAs to minimise eventual bias observed by the occurrence of off-target effects.
Cells were transfected with 50 nM of two different siRNAs targeting PANTR1 (siRNA 1 #n507582, siRNA 2 #n507583; both from Ambion Silencer Select, Ambion, Austin, Texas, USA) according to the “Fast Forward protocol” of HiPerFect Transfection Reagent (Qiagen; Hilden; Germany).
Non-targeting negative control siRNA (siRNA #4390843; Silencer Select Negative Control No.1 Ambion Silencer Select, Ambion; Austin; TX; USA) and cell death control (siRNA #SI04381048, AllStars Hs Cell Death; Qiagen; Hilden; Germany) served as controls applied in the same setting as described above.

2.2.3 RNA Isolation

For the detection of PANTR1 and other mRNA, total RNA was isolated first using the acid guanidinium thiocyanate-phenol-chloroform extraction method. A Trizol protocol according to the manufacturer's instructions (Invitrogen; Carlsbad; CA; USA) was used.

This method was first described in 1987 by Chomczynski et al. [146,147] and is a liquid-liquid extraction technique. When added to cells, they immediately lyze and two phases are generated: an aqueous phase, which contains RNA and DNA as well as an organic phase, which contains proteins. The aqueous phase is then transferred and isopropyl alcohol is added to start the precipitation process of the RNA. After centrifugation, RNA can then be visualised as a white, gel-like pellet at the bottom of the tube. The supernatant is extracted and the pellet is washed several times in 75% ice-cold ethanol. The RNA pellet is extracted and dried. It is then brought into solution again in RNAse free water for further processing.

To quantify the amount of isolated RNA, the concentration was measured with a spectrophotometer (Nanodrop 2000c, ThermoFischer Scientific; Vienna; Austria). RNA was then stored at -80°C until further processing.

2.2.4 cDNA Synthesis

For further experiments (e.g. qt-PCR) the extracted RNA needs further processing.
Briefly, in this step

1.) eventually still existing genomic DNA is removed,
2.) existing RNAses are inhibited and
3.) complementary DNA (cDNA) is created with the help of
    - various primers,
    - reverse transcriptase and
    - deoxyribonucleotide triphosphates.

We used the QuantiTect Reverse Transcription Kit (Qiagen; Venlo; The Netherlands) to synthesize cDNA from 1 µg of RNA according to the manufacturer's instructions.

2.2.5 Quantitative Polymerase chain reaction

To quantify the expression of different target genes with and without PANTR1 knockdown quantitative real-time polymerase chain reaction (qRT-PCR) was performed.

Primers were designed und purchased from Eurofins Genomics (Vienna, Austria). All specific primer sequences are listed in Table 5.

Quantification of lncRNAs and mRNAs was performed using the QuantiTect SYBR Green PCR kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's recommendations on a real-time PCR system (LightCycler 480, Roche, Mannheim, Germany).

LncRNAs were normalized to U6 (Eurofins Scientific, Vienna, Austria) and protein-coding genes to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; by Eurofins Genomics, Vienna, Austria).

Differences in expression were calculated in Excel (Microsoft Corporation; CA; USA) using the $2^{-\Delta\Delta Ct}$ method.

Table 5: List of all specific primers used in qRT-PCR

Gene	forward	reverse
PANTR1	CAT CAG GGG AGC AAC GTG AA	AGA GGA TGT GGT CAC TCC AGA
E2F1	CAT CAG TAC CTG GCC GAG AG AGT CTG AGT	CCC GGG GAT TTC ACA CCT TT
CDK6	CTG ATT ACC TGC TCC GC	TCC AGA ATC ATT GCA CCT GAG
CCNB1	TGG TGA ATG GAC TGT CAA GAA CA	TGC CAC AGC CTT GGC TAA AT
MKI67	CTG ACC CTG ATG AGA GTG AGG GA	GGG CTT CTC CCC TTT TGA GAG
MDM2	TCT TGA TGC TGG TGT ATA TCA AGT	TGA AGT GCA TTT CCA ATA GTC AGC
LAMC2	TGG ATG CAG TAC AGA TGG TGA TT	CCA GCC CCT CTT CAT CTA CAC
VEGF-A	CTC AGG GTT TCG GGA ACC AG	GTC GAT GGT GAT GGT GTG GT
GAPDH	AAG GTC GGA GTC AAC GGA TTT	ACC AGA GTT AAA AGC AGC CCT G
U6	CTC GCT TCG GCA GCA CA	AAC GCT TCA CGA ATT TGC GT

2.3 Caspase 3/7 apoptosis assay

Apoptosis is one form of self-programmed cell death and is a natural regulator to overcome the indefinite life of cells [148]. This essential cell process is mediated by the caspases 2 to 10. Caspases 3 and 7, execute the process after activation by merging their two subunits [149].

To investigate the impact of PANTR1 on apoptosis, a luminescent caspase 3/7 assay was performed. The principle behind this method is the quantification of emitted light due to the reaction described below.

In detail, cells are lysed and a luciferin in conjunction with original target sequence of caspases 3 and 7 – the tetrapeptide sequence DEVD (Aspartic Acid - Glutamic Acid - Valine - Aspartic Acid) - is added. DEVD is split off the complex by the caspases once entered in the cell. This luciferin emits light at a certain wavelength, which is measured. The amount of emitted light proportionally corresponds to the activity of caspases 3 and 7.

All three ccRCC cell lines ($5x10^3$ cells for RCC-MF, RCC-FG and $3x10^3$ cells for 769-P) were seeded into 96-well culture plates with growth medium and transfected according to the manufacturer's instructions as explained above.

Caspase Glo 3/7 reagent (Caspase-Glo 3/7 assay-Promega; Madison; WI; USA) was applied. Luminescence was recorded with a luminometer (LumiStar; BMG Labtech; Ortenberg; Germany) 72 and 96 hours after transfection.

All experiments were performed in quadruplicates. Differences in luminescence between the transfected cell lines and the negative control with full PANTR1 function were analysed using the ANOVA method.

2.4 WST-1 cell proliferation assay

To investigate the impact of PANTR1 on proliferation a calorimetric cell growth, WST 1-based assay was performed.

WST-1 proliferation assay is based on the following principle: The tetrazolium salt WST-1 (4-***3-(-Iodophynal)-2-(4-nitropehnyl)-2H-5-tretrazolio)1,3-benzene disulfate) is transformed to formazan by cellular enzymes. The more cells are viable, the more formazan is formed. This leads to higher light emission which is measured. This test works for living cells only and was first described by Mosmann *et al.* [150].

$5x10^3$ cells (for cell lines RCC-MF, RCC-FG) and $3x10^3$ cells (for cell line 769-P) were seeded onto 96-well culture plates. Cell death control samples as described above were used as positive control. Transfection was performed with 50 nM siRNAs as explained above. Cells were incubated with 200 µl of growth medium for a total of 96 hours. Repeatedly, every 24 hours WST-1 reagent was applied. Colorimetric changes were measured with a multi-plate spectrophotometer (SPECTROStar Omega, BMG Labtech, Ortenberg, Germany) at a wavelength of 450 nM (reference wave length of 620 nM).

Differences between the negative control (cells without previous treatment), the cell death control and the two corresponding transfected cell lines were analysed using the ANOVA method.

2.5 Scratch Assay

To assess one certain feature of proliferation, the wound healing capacity was investigated with a scratch assay. For this purpose, all three RCC cell lines were seeded on the outer 4 fields of a 6-well plate. Cell were either treated with a scramble RNA as negative control or with the two siRNAs as explained above. Plates were than scratched with the S*cratchmaster 3000*, a self-created device that ensures a scratch at a constant width and length compared to free-hand scratches. Therefore, an Eppendorf pipette with a 1000 µl tip was fixed in a 60° angle and the plate was then moved under the tip along a preformed master plate.

Cells were the incubated in 37°C atmosphere at 5% CO_2. After different waiting intervals (adjusted for each cell line), images were taken using an inverted light microscope (Olympus IX71). Images were analysed with Fiji software (open-source) with the “Wound-Healing-Tool plugin” to ensure objective and non-operator dependent analysis (for an example, see Figure 2). The scratched areas were automatically measured and possible statistical differences were then analysed using the ANOVA-method.

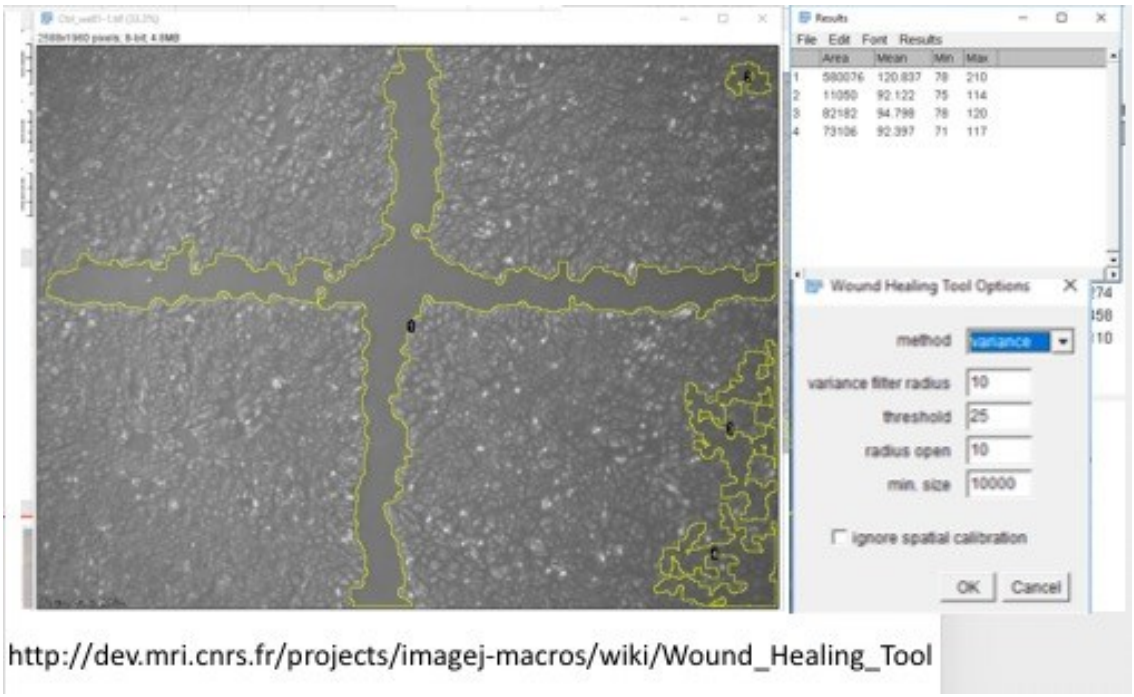


Figure 2: Example of measurement of the scratched/healed area in the scratch assay with the Wound-Healing-Tool-Plugin in ImageJ/Fiji-Software.

2.6 Endothelial cell migration assay

Angiogenesis is an essential step for tumour growth and is a very important feature of carcinogenesis. It represents the next hallmark of cancer which was investigated by several methods due to its importance in ccRCC.

First, an endothelial cell migration assay in HUVECs after siRNA-mediated knockdown of PANTR1 compared to HUVECS treated with a negative control was performed.
The QCM^{TM} 3 µm Endothelial cell migration assay (EMD Millipore/Merck group, Darmstadt, Germany) is based on the classical Boyden chamber with a membrane with a pore diameter of 3 µm. This size is optimal for HUVECs, but not sufficient for most other cells (e.g. fibroblasts) to ensure specific endothelial cell migration. In this assay, cells are attracted by fibronectin with which the floor of the well is coated. After migration, migrated cells are extracted, stained and quantified.

In detail, HUVECs were seeded on 60 mm plates and then transfected with 50 nM siRNA of both siRNA targeting PANTR1 for 48 h (n=3 for each). Colorimetric changes were measured with a spectrophotometer (SPECTROStar Omega, BMG Labtech, Ortenberg, Germany) at a wave length of 570 nm.

Differences between the two corresponding transfected cell lines and the negative were calculated using the ANOVA method.

2.7 Tube formation assay

The Millipore *In Vitro* angiogenesis Kit (Millipore, USA, obtained from Merck, Vienna, Austria) applied on HUVECs was used to generate a measurable, objective and reproducible angiogenesis model after siRNA-mediated knockdown of PANTR1 was performed.

HUVECs at a density of $1.5x10^4$ cells per well were seeded on a 96-well plate after a base lamina of ECMatrix^{TM} was applied. This matrix is a gel composed of basement proteins from Engelbreth-Holm-Swarm mouse tumours containing laminin, collagens, proteoglycan, heparane sulphate, entactin and nitrogen as well as several growth factors and enzymes. The cell-matrix-solution was the incubated for 16 hours as 37°C in an atmosphere of 5% CO_2. Three areas per well were randomly selected and photographed with an inverted light

microscope (Olympus IX71). All images were analysed with Fiji/ImageJ software (Fiji software/open-source). Several parameters such as number of junctions, nodes, meshes, extremities and branches as well as total branch length were analysed.

As data was parametric and as no measurable results for siRNA2 due to its strong effect could be obtained, T-Test was performed to reveal significant differences between the negative control and the siRNA.

2.8 Statistical analysis

Mean/median values and standard deviation (SD) were calculated and annotated as xy±SD. P values below 0.05 were regarded as significant and marked by an asterisk (*). P values <0.01 and <0.001 were asterisked when applicable with (**) and (***), respectively. The paraphrase “highly significant” or comparable was not used.

Unless otherwise stated, calculations were performed with Prism version 5.0 and/or 9.0 (GraphPad Software, San Diego, USA) and Microsoft Excel (Microsoft Corporation, Redmont, USA). If applicable, other statistical methods and programs that were used are annotated in the corresponding paragraphs.

2.9 Graphics

All graphical elements used in this manuscript are

- either downloaded from sources under the Creative Commons licence and then marked and adapted appropriately
- or drawn by the author himself with the illustration software GIMP 2.1 (Open-source).

3 Results

3.1 *In-silico* expression analysis of PANTR1

3.1.1 PANTR1 expression in different types of non-cancerous human tissue

PANTR1 expression from the NCBI Entrez gene database [121] was analysed across 95 different human individuals in 27 different tissue types (for detailed graphical illustration, see Figure 3).

PANTR1 expression was found at high levels in kidney and brain tissue. For brain tissue, 3 samples were analysed with a mean reads per kilobase per million reads placed (RKPM) of 2.25±1.1 and for kidney tissue, 3 samples were analyses with a mean RKPM of 1.55±0.62.

In all other tested human tissues, neglectable to no PANTR1 expression was measured.

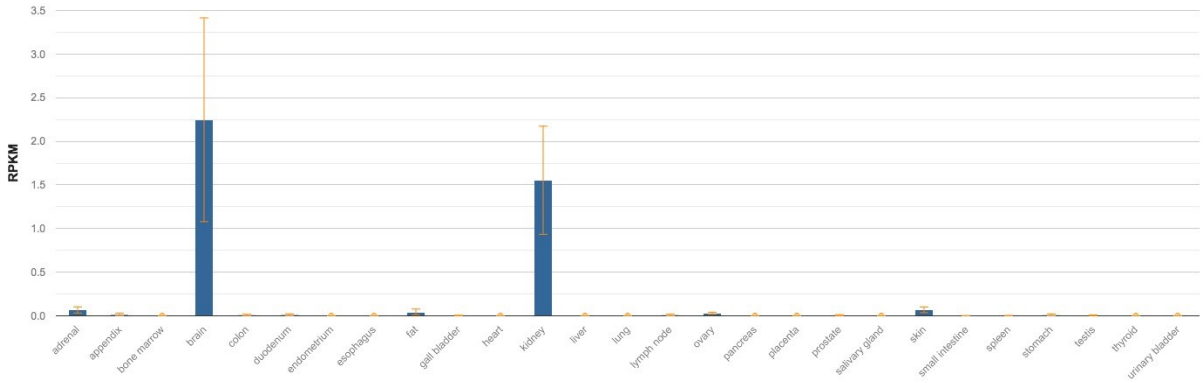


Figure 3: PANTR1 expression analysis across 95 different human individuals in 27 different tissue types (scaled in reads per kilobase per million/RKPM). From [121].

3.1.2 PANTR1 expression during human foetal development

PANTR1 expression data during human foetal development was also extracted from the NCBI Entrez gene database [121]. PANTR1 expression only reaches relevant levels in three human tissues: kidney, brain and heart.

In kidney tissue, the highest peak arrives in week 10 with a constant decline until week 20. After week 20, there is no data on PANTR1 expression in human kidney tissue.

In heart tissue, there is only one peak in week 10 with no further expression.

Human brain tissue also constantly expresses PANTR1 with no timeline information (for detailed graphical illustration, see Figure 4).

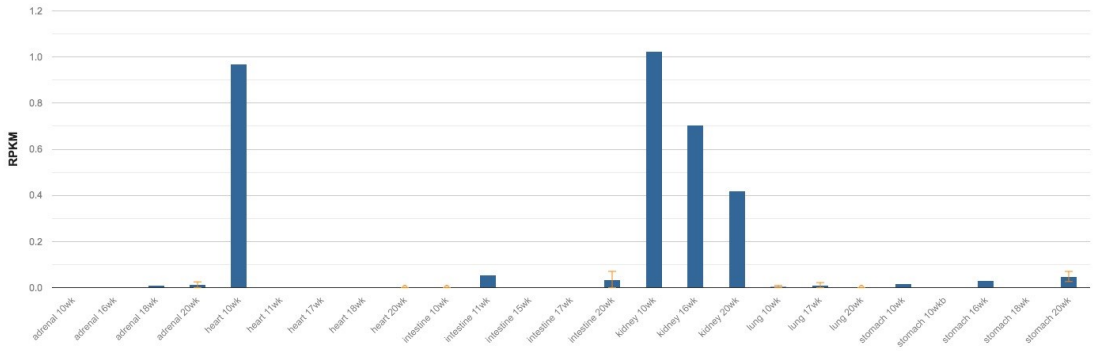


Figure 4: RNA sequencing data showing the expression of PANTR1 in different human foetal tissues across different weeks of gestation (scaled in reads per kilobase per million/RKPM). From [121].

3.1.3 PANTR1 expression in different types of human cancer

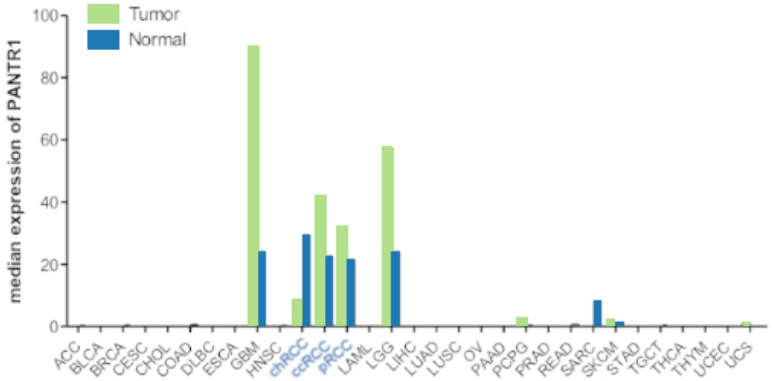
This data regarding PANTR1 expression in different types of human cancers was extracted from the GEPIA database [143,144].

PANTR1 is expressed in only a few human cancer tissue types: ccRCC, chRCC, pRCC, glioblastoma multiforme and low-grade glioma, the two latter being malignant brain tumours (for detailed graphical illustration, see Figure 5).

For ccRCC, median expression in cancer tissue is higher compared to the corresponding normal kidney tissue (median expression 41.25 vs 22.36 RPMK).

For pRCC, median expression is higher than in the normal kidney tissue with less dominant effects compared to ccRCC (median expression 31.57 vs. 21.31 RPMK).

For chRCC, the expression profile is contrary to its RCC counterparts ccRCC and pRCC: Median expression is lower in cancer tissue compared to the corresponding normal kidney tissue (median expression 8.08 vs 29.2 RPMK).



median expression of PANTR1		
sample type	Tumor	Normal
GBM	89,39	23,78
chRCC	8,08	29,2
ccRCC	41,25	22,36
pRCC	31,57	21,31
LGG	57,2	23,78

Figure 5: RNA sequencing data of PANTR1 expression in different kinds of human cancer tissues (in green) matched with matched normal tissue (in blue). Modified after [1] *under Creative Common License 4.0, originally downloaded from and published by* [143,144]. *GBM: glioblastoma multiforme; chRCC: chromophobe RCC; ccRCC: clear-cell RCC; pRCC: papillary RCC; LGG: brain lower grade glioma. All other abbreviations are not relevant.*

3.1.4 Higher PANTR1 expression in cancerous vs. matched normal human kidney tissue

PANTR1 expression data for 144 human samples (matched ccRCC vs. normal kidney samples, n=72 in each group) was extracted from NCBI's gene expression omnibus/GEO database.

Mean expression level was 1714±734 RPKM for ccRCC tissue compared to 835.7±548.2 RPKM for matching normal kidney tissue samples. The difference reached statistical significance with a p-value of <0.0001. Figure 6 shows a boxplot with direct graphical comparison.
Hence, PANTR1 expression is significantly higher in ccRCC tissue compared to matched normal kidney tissue samples in humans.

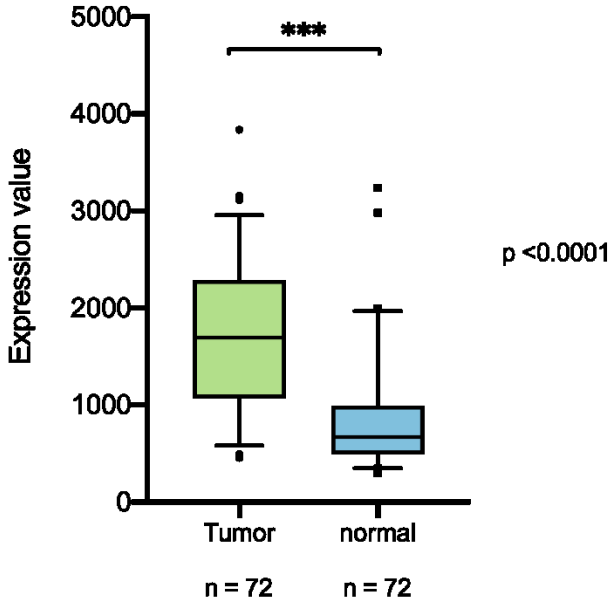


Figure 6: Histogram demonstrating PANTR1 expression in ccRCC versus normal kidney tissue in a matched cohort of 144 samples of ccRCC (in green) and normal kidney tissue samples (in blue; n=72 for each group). Data extracted from GEO Database.

3.1.5 Survival analysis stratified by PANTR1 expression

Disease-free survival (DFS) as well as overall survival (OS) was calculated after stratification of the patients into two groups according to the median PANTR1 expression at a cut-off of 50%.

For ccRCC, significant better OS (p=0.004, HR 0.65) but not DFS (p=0.078, HR 0.73) could be demonstrated in patients with high PANTR1 expression.

For pRCC and chRCC, neither DFS nor OS are significantly associated with PANTR1 expression (for all p>0.05).

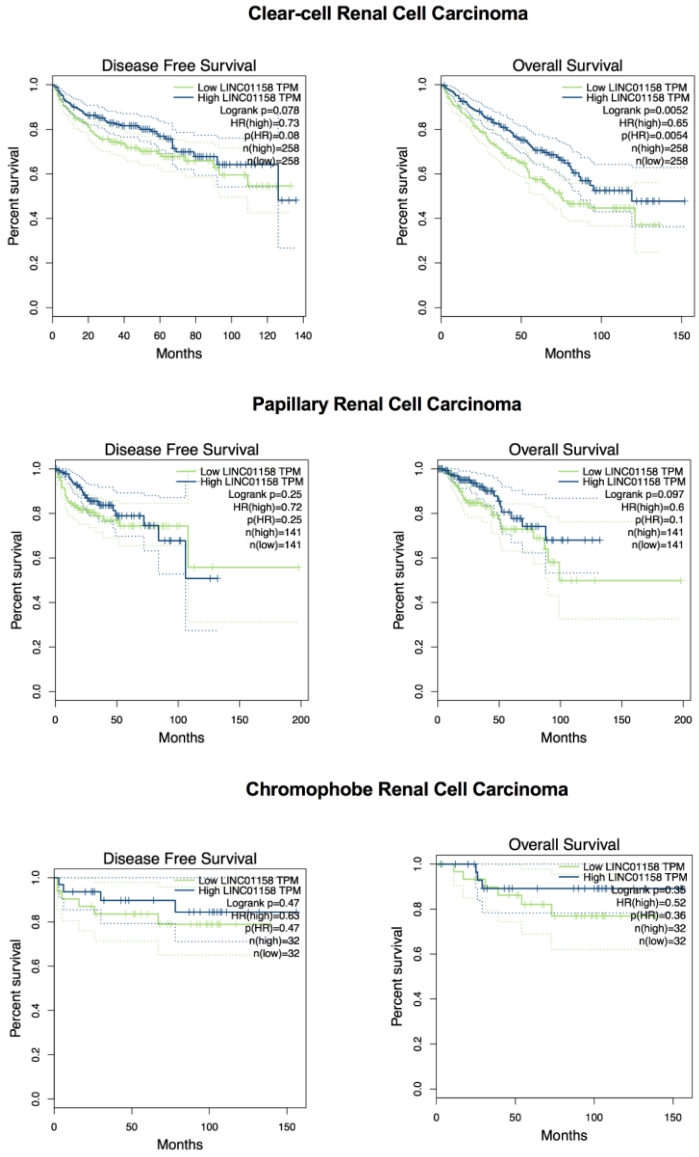


Figure 7: Disease-free and Overall Survival plots for ccRCC, pRCC and chRCC after stratification for a 50% cut-off of PANTR1 expression. Data extracted and plots generated with Gepia's integrated calculator (accessible under http://gepia.cancer-pku.cn/detail.php?clicktag=correlation).

3.2 Cell Culture experiments

After *in-silico* data had provided hints for a certain role of PANTR1 in ccRCC, *in-vitro* experiments were conducted to clarify its biological role.

Therefore, three different ccRCC cell lines and an endothelial cell line were chosen to investigate the effect of PANTR1 on the three following *hallmarks of cancer* [138,139]:

- Enabling replicative immortality and resistance to cell death/apoptosis
- Inducing angiogenesis
- Sustaining proliferative signalling

3.2.1 Confirmation of baseline PANTR1 expression in RCC cell lines

Before any loss-of -function experiment, PANTR1 expression was confirmed in all four cell lines (HUVEC, RCC-FG, RCC-MF,769-P, n=3 for each) with qRT-PCR. The expression levels of PANTR1 were almost at the same relative level with no significant differences in all three RCC cell lines.

Different expression levels could be demonstrated for the three RCC when compared to HUVECs (for all combinations $p < 0.0001$; one-way ANOVA) without any relevance for the following experiments.

Hence, all four cell lines did express PANTR1 at a sufficient level and could be used for further experiments.

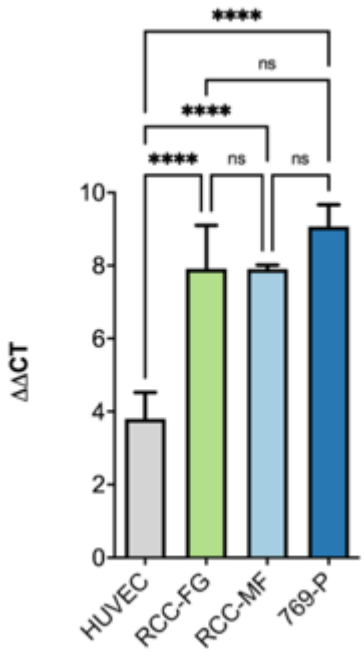


Figure 8: Column bar graph showing the mRNA expression levels of PANTR1 in comparison to the housekeeping gene in all 4 cell lines (n=3). ns = non-significant. Modified after [1] *under Creative Common License 4.0.*

3.2.2 Confirmation of siRNA-mediated knockdown of PANTR1

Knockdown was performed with two different siRNAs against PANTR1.

Relative expression of PANTR1 in comparison to the negative control sample could be confirmed with statistically significant difference in each of the four cell lines and with each of the two siRNAs (p value for every single test between $p<0.01$ and $p<0.001$).

Thus, both of the two siRNAs against PANTR1 could be validated for a use in further studies in these four cell lines.

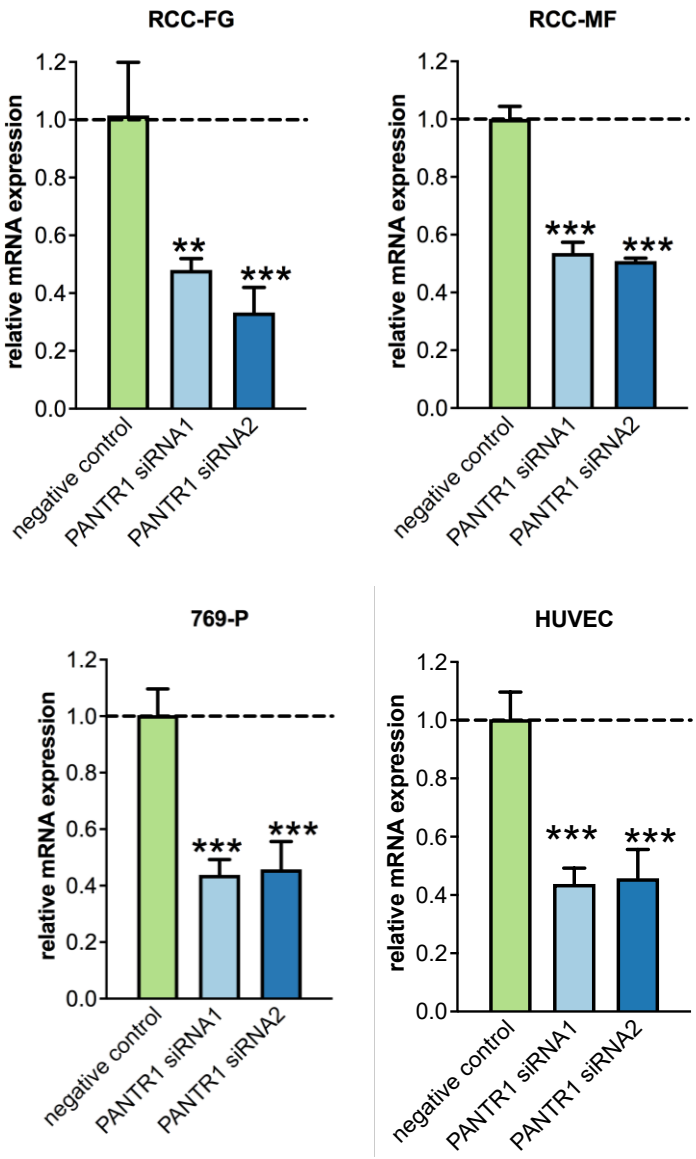


*Figure 9: Column bar graphs showing qRT-PCR-based validation of siRNA-mediated PANTR1 knock-down efficiency in all three RCC cell lines and HUVECs 48h after transfection (n=3); **p<0.01, ***p<0.001. From* [1] *under Creative Common License 4.0.*

3.2.3 Effects of PANTR1 on cellular growth

3.2.3.1 PANTR1 has a significant effect on cellular growth and viability

To assess effects of PANTR1 on cellular growth, a WST1 assay was performed.

A consistent and statistically significant (***p<0.001) effect can be seen for all three cell lines for the latest measured time point at 96 h. After 72 h, a statistically significant effect could be seen in RCC-FG and RCC-MF cell lines. The most persistent and clear effect could be observed for RCC-MF, where analysis of the cell lines also revealed a significant difference after 48 h.

Throughout all cell lines, cell death control worked as a positive control for cell death with minimal absorbance at 96 h.

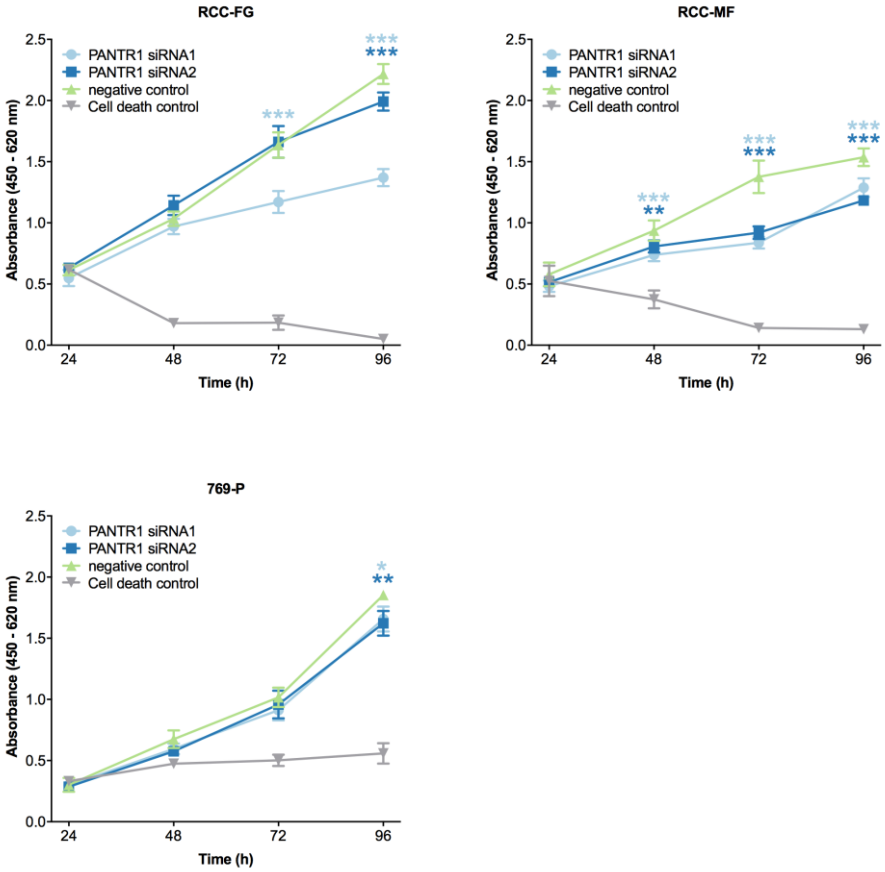


*Figure 10: Multiple line graph demonstrating absorbance for RCC-FG, RCC-MF and 769-p at 24, 48, 72 und 96h after treatment. n=3. *p<0.05, **p<0.01, ***p<0.001. Modified after* [1] *under Creative Common License 4.0.*

3.2.3.2 PANTR1 has no constant effect on proliferation genes

In order to find differentially expressed proliferation markers, a corresponding gene panel was compiled and analysed with qRT-PCR.

For RCC-FG genes for E2F, MCM2 and CDK6 were found to be of significant difference in relative expression (for details, please see Figure 11).

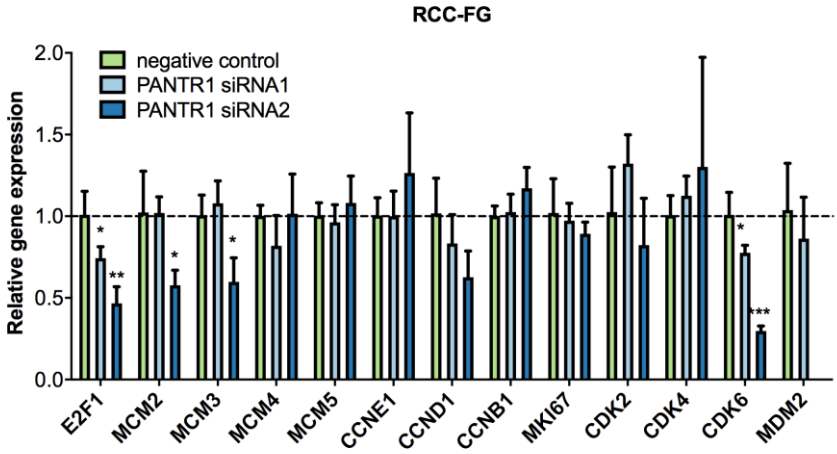


Figure 11: Column bar graph showing relative mRNA expression. (relative to the negative control) for a proliferation gene panel in RCC-FG cell line. n=3.

For RCC-MF genes for CCNB1, MKI67 and MDM2 were found to be differentially expressed at a significant level (for details, please see Figure 12).

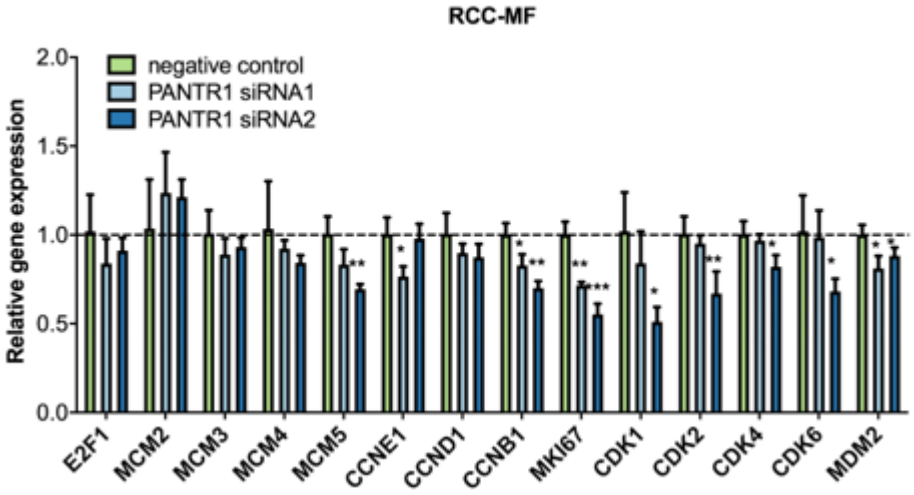


*Figure 12: Column bar graph showing relative mRNA expression. (relative to the negative control) for a proliferation gene panel in RCC-MF cell line. n=3. *p<0.05, **p<0.01, ***p<0.001.*

For 769-P no significant difference compared to the negative control was found (for details, please see Figure 13).

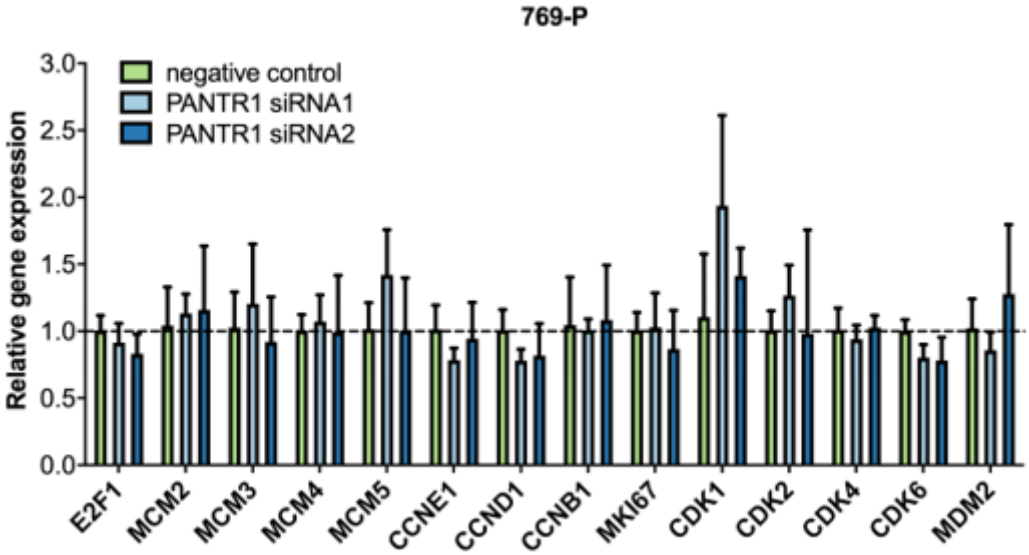


Figure 13: Column bar graph showing relative mRNA expression. (relative to the negative control) for a proliferation gene panel in RCC-MF cell line. n=3.

Unfortunately, there was no consistent effect that could be demonstrated in all 3 three RCC cell lines on mRNA levels.

3.2.4 PANTR1 decreases apoptotic activity

3.2.4.1 Caspase 3/7 apoptosis assay

As poof of principle of the assay in this setting, the three untreated RCC cell lines were analysed in comparison to a cell death control (n=4 for each group).

Results are illustrated in Figure 14 and show a significant difference ($p<0.001$ for all) between the negative control and the cell death control samples.

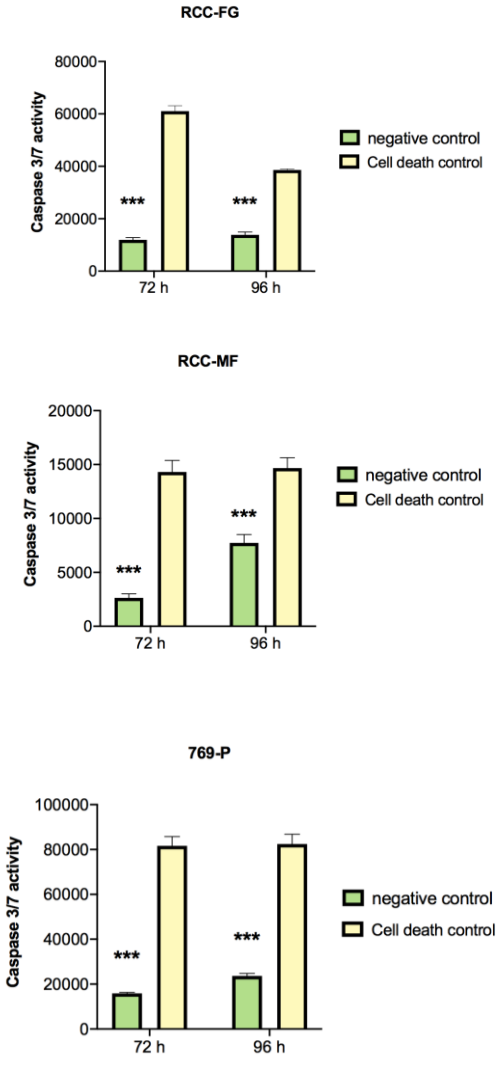


*Figure 14: Column bar graphs showing the caspase 3/7 activity in 2 different groups: control group (without any treatment) vs. cell death control after 72h and 96h after treatment. n=4; mean±SD. ***p<0.001.*

As a next step, negative control samples were compared to knockdown cells treated with the 2 different siRNAs.

In each of the three RCC cell lines, siRNA-mediated PANTR1 knockdown resulted in significantly higher detection of the luminescent which equals higher caspase 3/7 activity (p-value of at least of <0.01). For illustration und further details, please see Figure 15.

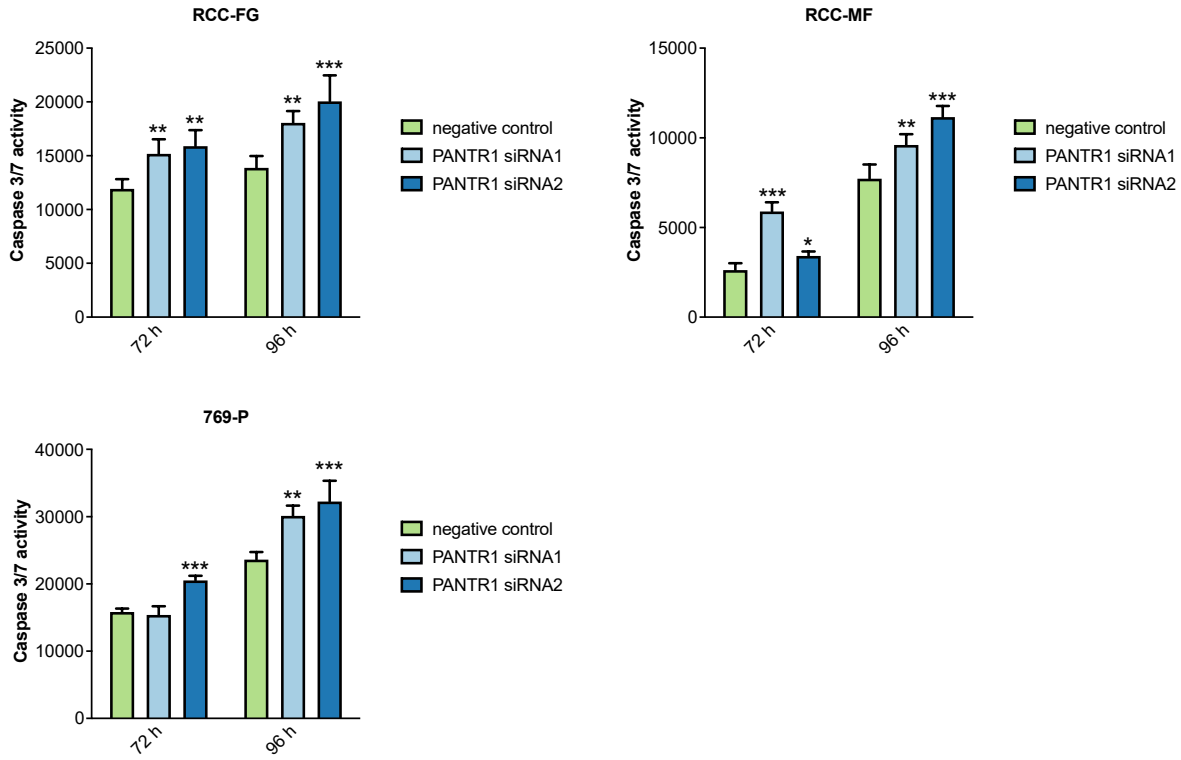


*Figure 15: Column bar graphs demonstrating caspase 3/7 assay either under control conditions (negative control siRNA) or after siRNA-mediated knock-down of PANTR1 72 or 96 h after transfection. n=4; mean ± SD. *p<0.05, **p<0.01, ***p<0.001. From* [1] *under Creative Common License 4.0.*

3.2.5 PANTR1 does not influence cancer cell migration

To assess whether PANTR1 is able to influence cancer cell migration, a classical scratch assay was performed in the three ccRCC cell lines. Exemplary images of each cell line at each time point are depicted in Figure 17.

PANTR1 had no constant effect on all three cell lines. The measured areas between the negative control and the two siRNAs against PANTR1 after individual time points differed at a statistically significant level in RCC-MF (negative control vs siRNA1: $p<0.05$, Negative Control vs siRNA2: $p<0.01$).
For RCC-FG, no statistically significant difference could be measured ($p>0.05$ for all combinations).
For 769-P, a highly significant effect for the remaining areas could be observed between the negative control and siRNA2 ($p<0.001$) but not between the negative control and siRNA1 ($p>0.05$) (For details, please see Figure 16).

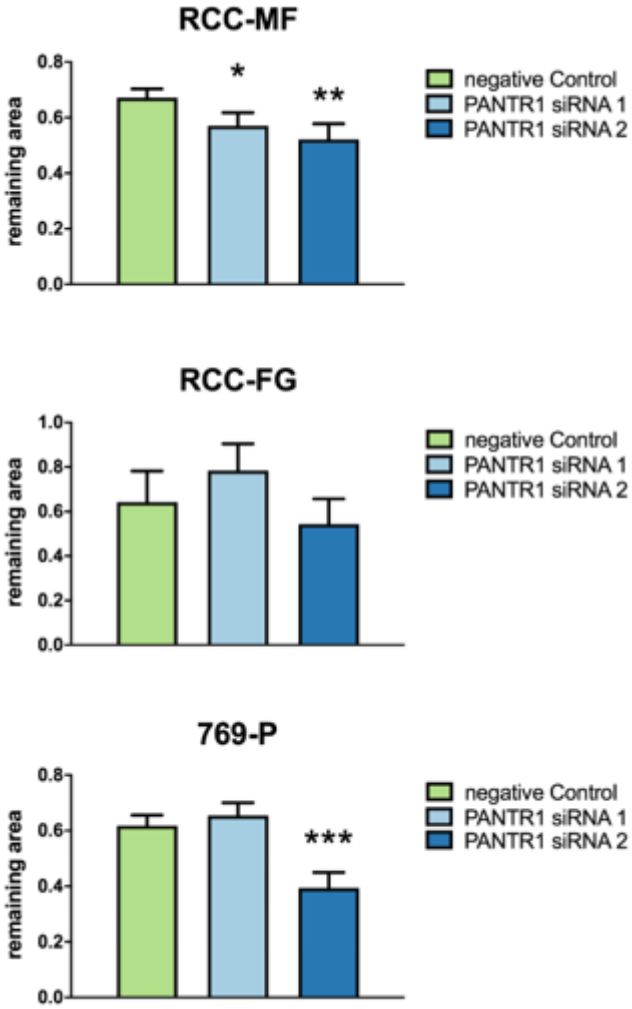
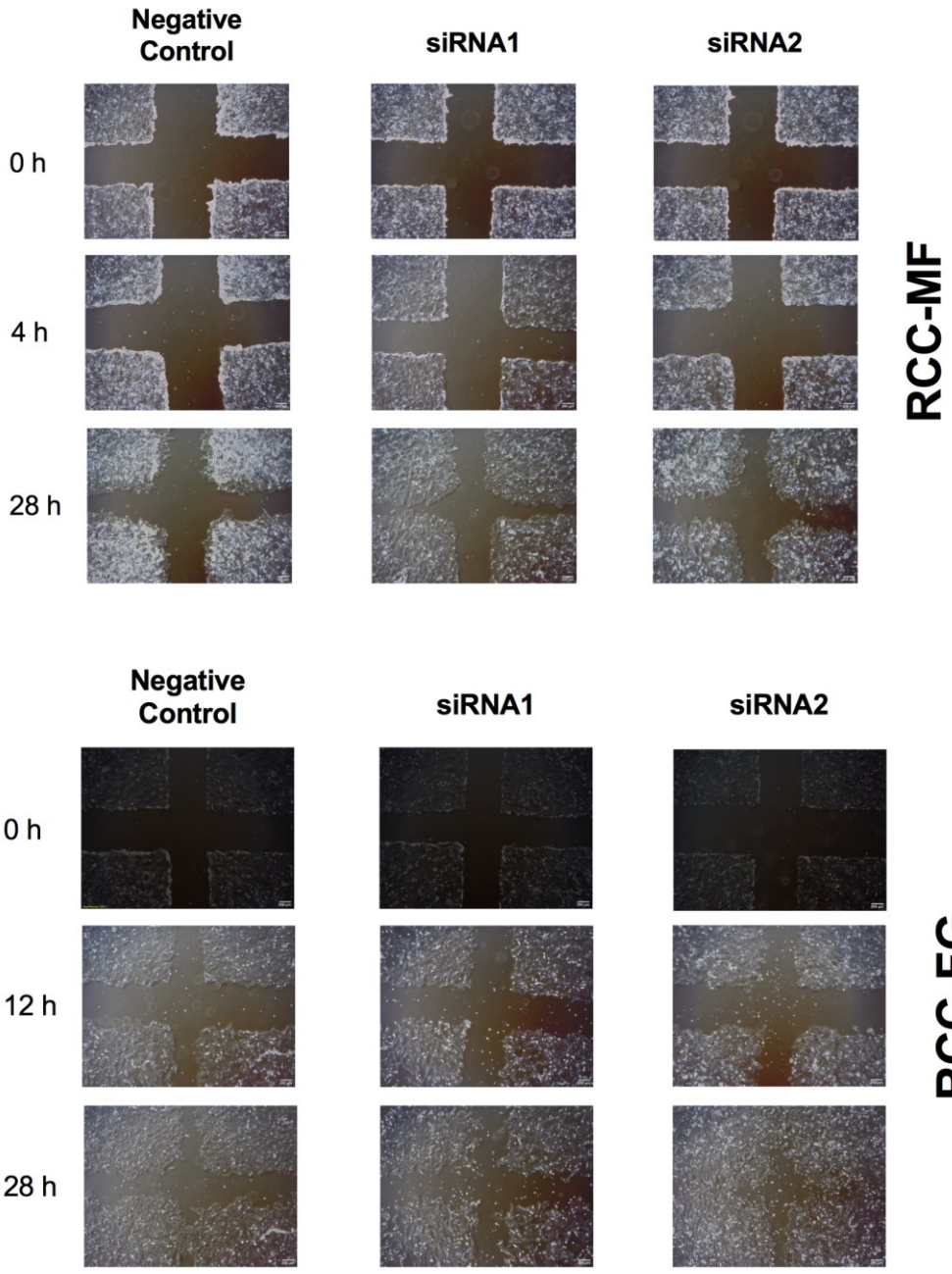


*Figure 16: Column bar graph showing the remaining areas at the last point in time in each of the three RCC cell lines. n=3. *p<0.05, **p<0.01, ***p<0.001.*

To conclude, there might be an effect of PANTR1 on wound healing capacity but this effect could not be demonstrated in our experiment in all of these three cell lines.


Negative Control
siRNA1
siRNA2
0 h
4 h
28 h
RCC-MF
Negative Control
siRNA1
siRNA2
0 h
12 h
28 h
RCC-FG

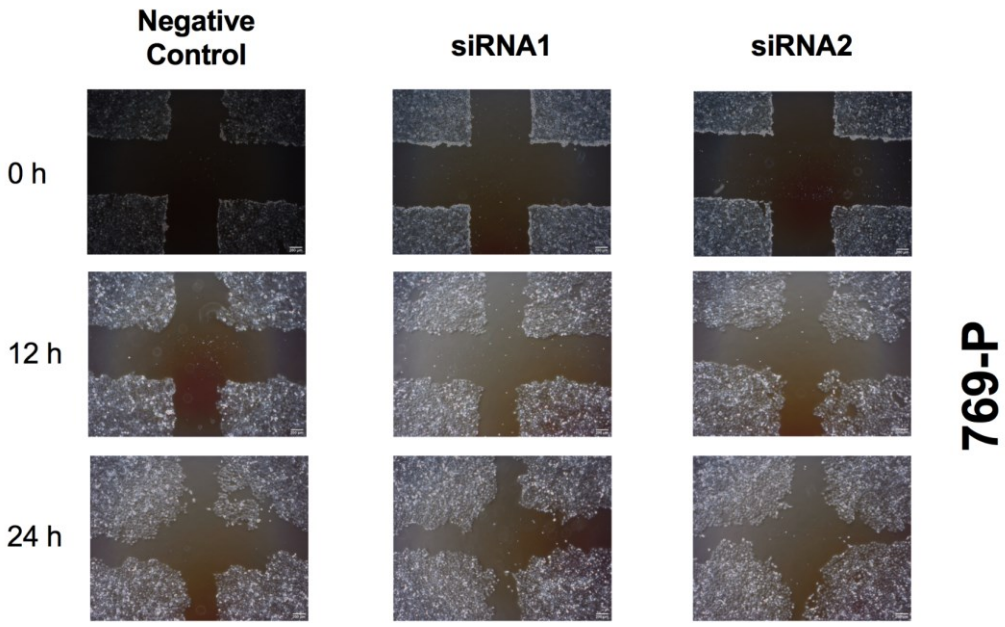


Figure 17: Exemplary picture of each cell line with no treatment (negative control) or after siRNA-mediated knockdown with the two siRNAs at 3 different time points imaged for scratch assay. n=3 for each.

3.2.6 Effects of PANTR1 on angiogenesis

3.2.6.1 PANTR1 positively influences endothelial cell migration

One crucial step towards excessive tumour growth is the formation of new vessels to provide oxygen and nutrients, also known as vasculo/angiogenesis.
As a first step, endothelial cell migration with or without the influence of PANTR1 was investigated.

The siRNA-mediated knockdown of PANTR1 resulted in significantly decreased endothelial cell migration compared to the negative control ($p<0.05$ for siRNA1 and $p<0.01$ for siRNA 2. For further details, please see Figure 18.

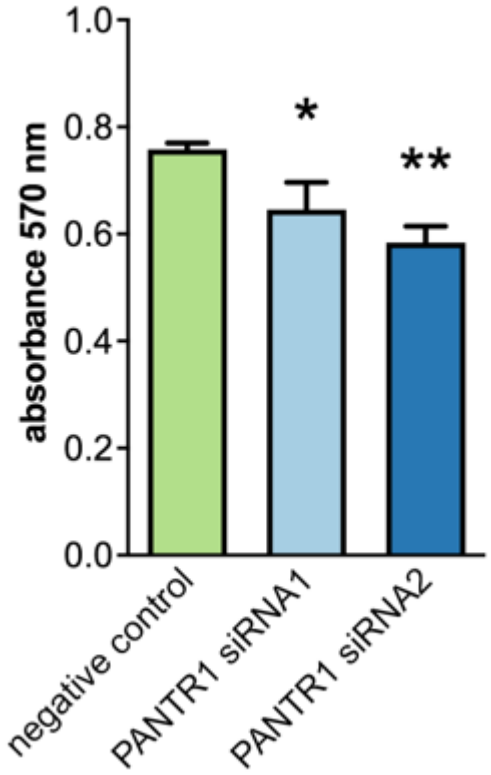


*Figure 18: Column bar graph demonstrating the results of the endothelial cell migration assay with HUVECs (negative control vs siRNA-mediated knockdown. n=3. Mean±SD. *p<0.05, **p<0.01. Taken from* [1] *under Creative Common License 4.0.*

3.2.6.2 PANTR1 has a significant effect on several blood vessel parameters

For further proof of relevance of PANTR1 regarding angiogenesis, another crucial step for angiogenesis was investigated: the new formation of blood vessels, also called vasculogenesis.
Therefore, a tube formation assay was conducted (n=5) and 6 exemplary parameters were investigated: number of junctions, nodes, meshes, extremities and branches as well as total branch length.

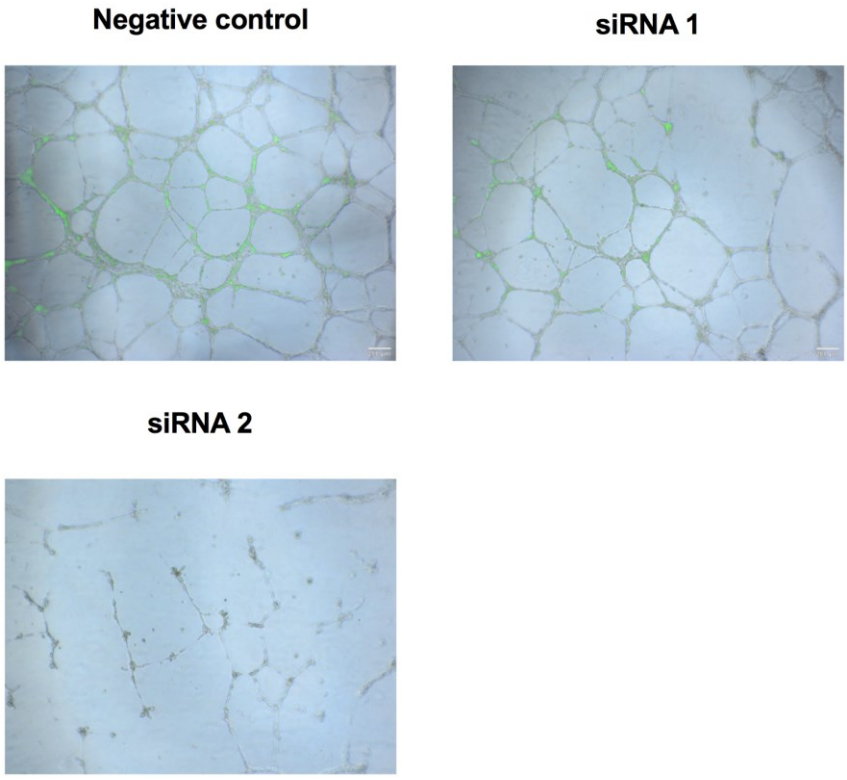


Figure 19: Exemplary and randomly taken pictures during the tube formation assay of the negative control, siRNA1 and siRNA2. Modified after [1] *under Creative Common License 4.0.*

The effect of siRNA2 regarding all parameters was so strong that analysis software could not measure the parameters. Therefore, no significance can be noted. The effect can clearly be seen in Figure 19.

Significant differences between siRNA1 and the negative control could be observed for numbers of junctions ($p<0.05$), branches, meshes and nodes (for all 3, $p<0.01$). The difference for length of branch and number of extremities was of no statistical significance ($p>0.05$).

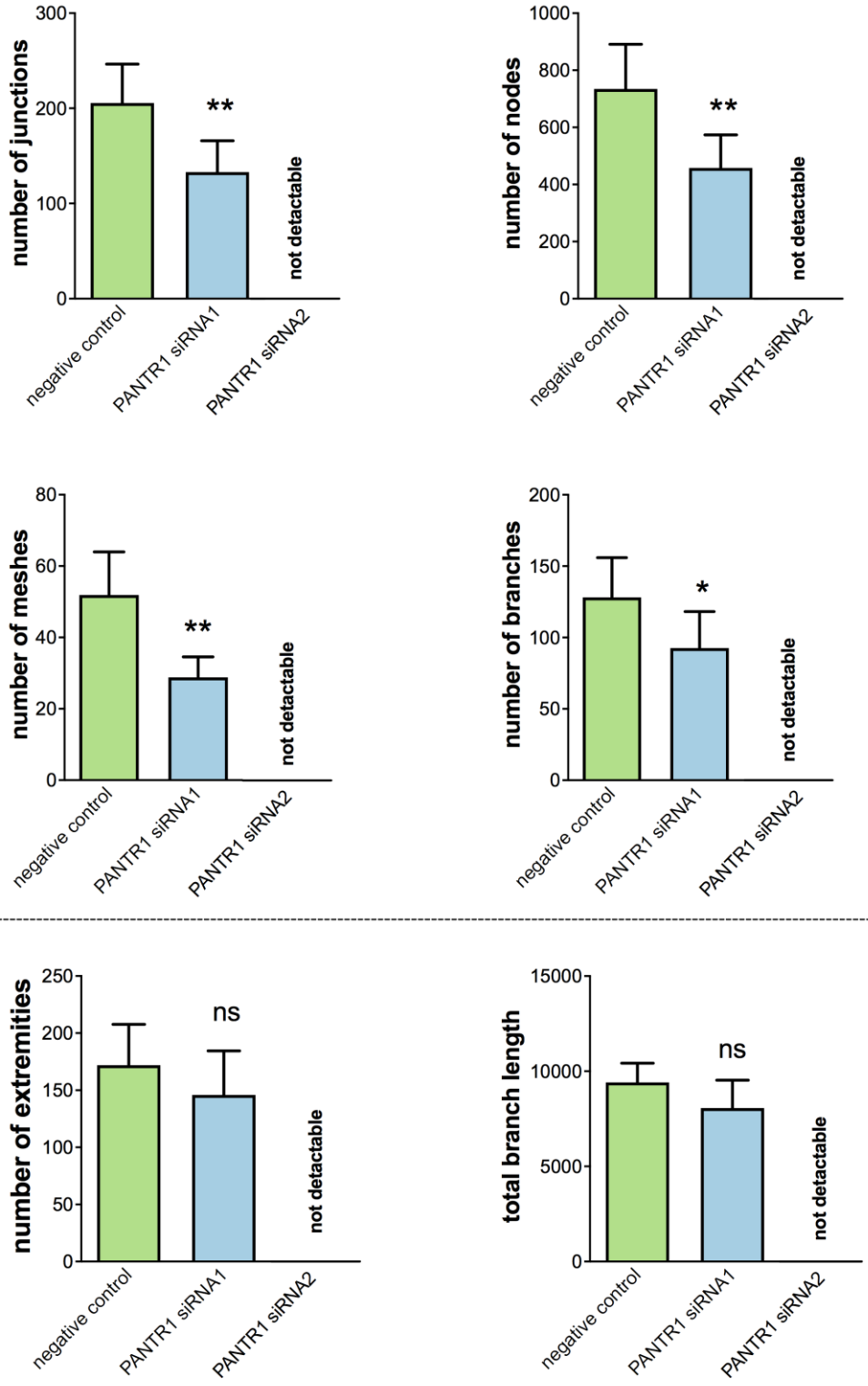


*Figure 20: Column bar graph showing number of junctions, nodes, meshes, extremities and branches as well as total branch length as results of analysis of the tube formation assay (negative control, after treatment with siRNA 1 and 2, n= 5 for each). ns=not significant, *p<0.05, **p<0.01. The 4 upper graphs extracted and modified from* [1] *under Creative Common License 4.0.*

3.2.6.3 PANTR1 influences the gene expression pattern of angiogenetic genes

In order to find differentially expressed genes, a primer panel of angiogenetic genes was designed and analysed with qRT-PCR.

For RCC-FG cell line, only 2 genes were found to be expressed differently at a significant level by both siRNA compared to negative control: LAMC-2 and VEGF-A.

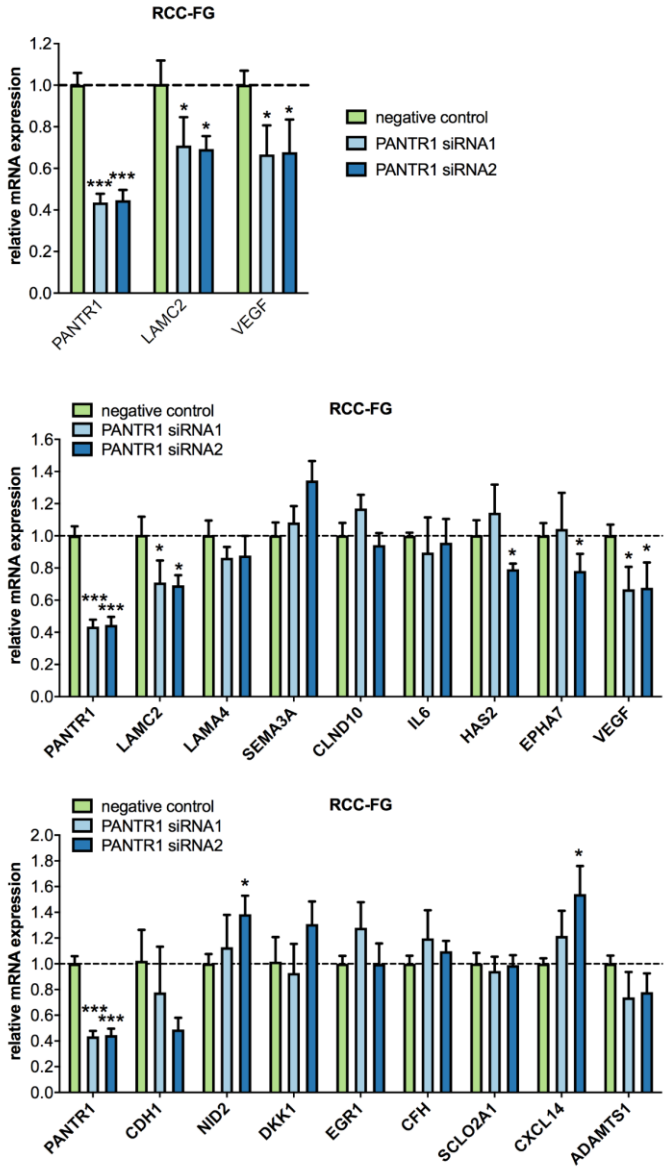


*Figure 21: Column bar graphs demonstrating relative mRNA expression levels of angiogenesis-associated genes after PANTR1knock-down in RCC-FG cells; n=3. *p<0.05, **p<0.01, ***p<0.001.*

3.2.6.4 Significant correlation between PANTR1 and VEGF-A

In qRT-PCR of the angiogenesis panel, we found LAMC2 and VEGF-A to be upregulated in ccRCC tissue. To possibly correlate this to PANTR1, a correlation analyses between these two and PANTR1 based on GEPIA server data was performed.

PANTR1 and VEGF-A

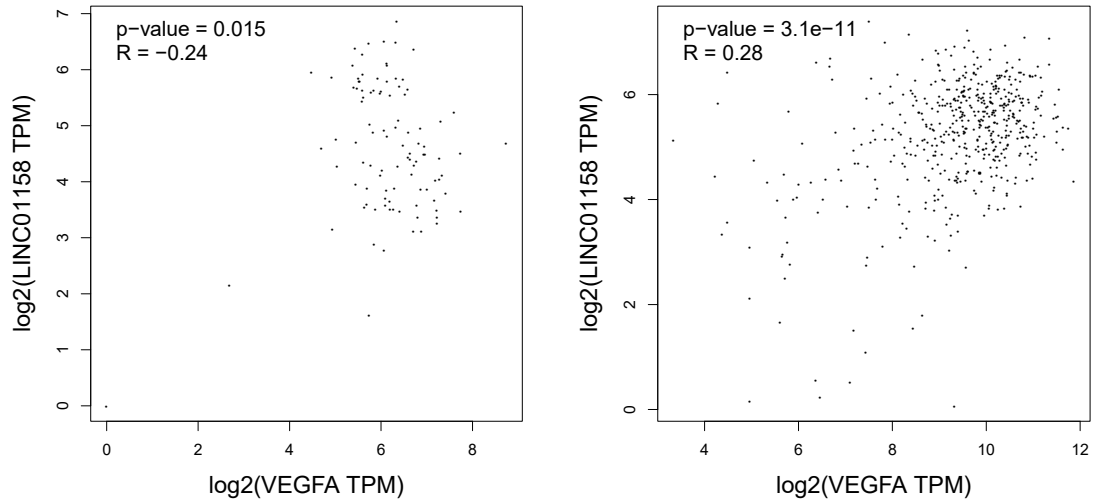


Figure 22: Scatter plots of regression analysis between VEGF-A and PANTR1. Left plot: normal kidney tissue (TCGA and GTEx), right plot: ccRCC tissue (TCGA).

Regression analysis of VEGF-A and PANTR1 in TCGA and GTEx datasets revealed a significant correlation (p=0.015, spearman's rank test) with a correlation coefficient of R=-0.24 for normal kidney tissue. In ccRCC tissue (from TCGA), higher significance ($p=3.1x10^{-11)}$ as well as the correlation coefficient (R=0.28) also suggest a relationship in ccRCC compared to normal kidney tissue.

When comparing the scatterplots directly, one can observe that in ccRCC tissue dots seem to swarm around the ideal regression curve while bearing in mind that a lot more data points for ccRCC is available.

PANTR1 and LAMC-2

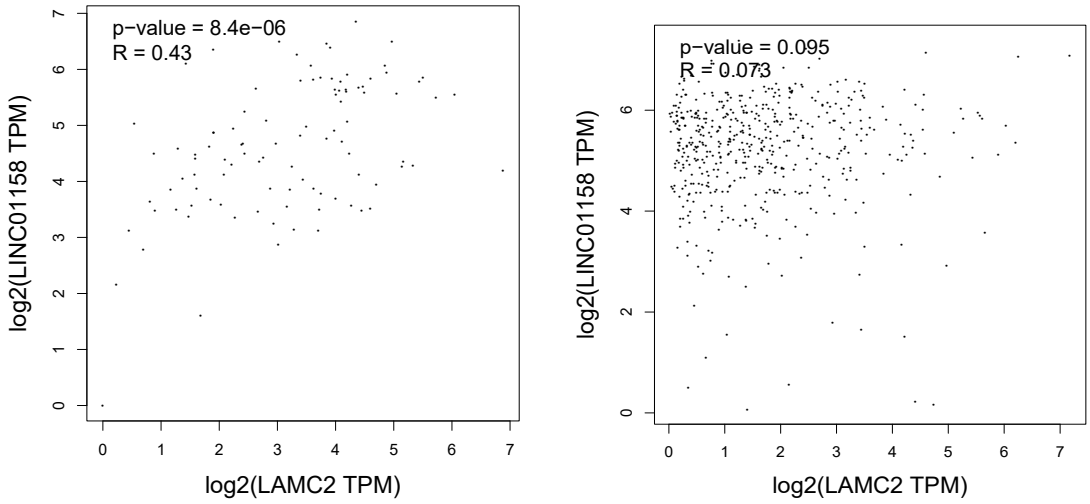


Figure 23: Scatter plots of regression analysis between PANTR1 and LAMC-2. Left plot: normal kidney tissue (TCGA and GTEx), right plot: ccRCC tissue (TCGA).

Regression analysis of LAMC-2 and PANTR1 in TCGA and GTEx datasets revealed a significant correlation ($p=8.4x10^{-6}$, spearman's rank test) with a correlation coefficient of R=0.43 for normal kidney tissue. In ccRCC tissue (from TCGA), no significance could be reached (p=0.095) and the correlation coefficient (R=0.073) almost reached 0.

4 Discussion

LncRNA PANTR1 is a non-coding transcript located about 4kb upstream of the POU3F3 gene on chromosome 2 [121]. PANTR1 was identified and has been investigated since for its role as an oncogene with a possible relation to POU3F in several types of cancer such as eSCC, glioma, prostate, cervical, colorectal, gastric and hepatocellular carcinoma as well as breast cancer.

This is the first study to demonstrate the oncogenic effects of PANTR1 in RCC/ccRCC.

4.1 The role of PANTR1 as a biomarker

Regarding ccRCC based on the results after dichotomization, high PANTR1 expression seems to be prognostic for longer DFS and OS compared to low PANTR1 expression. The results were calculated with GEPIA (Gene Expression Profiling Interactive Analysis), a web-based tool that was established based on GTEx and TCGA data. For neither of two other main subtypes of RCC, DFS nor OS differed significantly.

Data from 170 patients from Czech Republic were analysed by our group (not shown in this dissertation but in [1]) but we could not confirm these results. Eventually, low PANTR1 expression was predictive for longer DFS and OS in this data.
In contrast to the results from GEPIA database, the results from [1] are in full compliance with survival analysis data from other entities such as eSCC [128], triple-negative breast cancer [130], nasopharyngeal carcinoma [133] and cervical cancer [134].

In the end, we cannot be certain how the data was calculated by GEPIA or whether any database error somehow occurred. To conclude these conflicting results, we would rather suggest to use PANTR1 as a predictive biomarker for DFS and OS with regard to its oncogenic function. Low expression of PANTR1 seem to be predictive for longer survival but confirmatory results for ccRCC are needed.

4.2 POU3F3 - a link to carcinogenesis?

PANTR1's neighbour in human DNA is the protein-coding gene POU3F3, which is a well-known key player during the foetal development of the human central nervous system and the kidneys. POU3F3 homozygous knockout-mice display highly increased plasma parameters indicating decreased kidney function with accompanying developmental defects in the forebrain and hippocampus [107]. Further examinations revealed kidneys of smaller size [107,111]. In analogy to mice, humans with defective POU3F3 gene present with a broad spectrum of features which are in direct connection to the extent of the mutations. They span from mild cognitive impairment and mild dysmorphia to complex malformation of the kidney, anus, heart and corpus callosum [106,110].

The proximal tubule has long been postulated as the segment of the kidney, where ccRCC arises from. Undoubtedly, biomarkers (proteins like CD10, Villin, RCC antigen etc. and even mRNA signatures) that are expressed in the proximal tubule are also found to be expressed in ccRCC. Nevertheless, there are also markers of ccRCC, such as CD24, cytokeratin 19 or galectin 3, which are expressed by the distal tubule or collecting duct cells [151].

Thus, no final answer to this question can finally be given. Nevertheless, it therefore could be postulated that POU3F3 may play a role in carcinogenesis of RCC by its widespread influence during the foetal period as explained above.

4.3 PANTR1 as an epigenetic regulator of cancer?

Only one research group has yet identified a possible epigenetic regulation mechanism of PANTR1. Liu et at. [152] proposed possible interaction of PANTR1 with PCR2 of eukaryotic cells in their own eSCC cell lines. This seems reasonable as lncRNAs are known for their role as epigenetic regulators [153].

Epigenetic regulation means activation or deactivation of a certain gene without further modification of the DNA. This can be done via different ways - one important being through an enzyme named PCR2. PCR2 is currently known to affect at least 2000 different genes of at least 10 chromosomes. This protein contains 3 subunits: SUZ12, EED and (EZH1/2). The complex trimethylates H3K27 to initiate the H3K27me3 modification on nucleosomes. This

results in compaction of the chromatin to prevent transcription, and thus, deactivation of the gene. Methylation is primarily done by EZH2 with the help of the two other subunits. Easily said, PCR2 can act as an “on-off-switch” for genes and thus plays a major role in carcinogenesis when silencing tumour suppressor- or oncogenes [154].

Regarding ccRCC, the role of EZ2H2 was investigated separately in cancerous tissue and with the use of an *in-vitro* knockdown. Silencing of EZ2H2 results in tumour suppression regarding different hallmarks of cancer whereas overexpression is associated with advanced stage, poorer DFS and OS [118]. Thus, EZ2H2 seems to act as a silencer of tumour suppressor genes subsequently caused by overexpression in ccRCC [16].

Liu et al [152] found an association of PANTR1 with EZH2 in eSCC cells to point out possible epigenetic regulation of PANTR1 via the PCR2 complex. Furthermore, they could prove that overexpression of PANTR1 leads to hypermethylation of the CpG (**C**ytosine-**P**hosphate-**G**uanin) islands of the promoter region of POU3F3 and thus to overexpression of POU3F3 mRNA [153]. This might be an important fact as this certain CpG region was hypermethylated resulting in suppressed activity of POU3F3 in several different kinds of cancer (salivary gland [155], prostate [156] and lymphoma [118]). CpG Islands are regions within the genome where CpG dinucleotides occur more frequently than in other regions [153]. Further investigation on the role of POU3F3 in renal cell cancer are not be found, so possible protective anti-cancer activity of POU3F3 as a tumour suppressor can only assumed.

Regarding epigenetic regulation in ccRCC, current research results suggest a major involvement of the loss short arm of chromosome 3 as a prequel to subsequent development of ccRCC. This key event does not only involve the famous VHL gene but also several others are involved in histone or chromatin modification. Among them, BAP1 also is a so-called *two hit suppressor gene* where malfunction will only result after modification of both alleles. Casually, the same occurs to the VHL gene, which will be discussed below. BAP1 is another subunit of the above-mentioned PRC complex and is involved in histone modification such as SETD2 which is a histone-lysine-methyltransferase. The third gene/protein PRBM1 residing on chromosome 3p with influence on the SWI/SWF-complex (SWItch/Sucrose Non-Fermentable) which is also a chromatin/nucleosome remodelling complex [17].

To sum up the current state of knowledge on ccRCC carcinogenesis, disrupted epigenetic regulation of transcription is the key to formation of ccRCC in humans. PANTR1 may play a role through the influence of this regulatory complex.

4.4 Hallmarks of Cancer

PANTR1 has been suggested to exhibit a versatile role on multiple hallmarks of cancer in different kinds of cancer.

The so-called "*hallmarks of cancer*" were first formulated by Douglas Hanahan and Robert Weinberg in 2000 published in *Cell* [138] (with an update in 2011 [139]). These hallmarks represent a principle of categorization of the numerous characteristics of cancer cells to facilitate the understanding.

These principles are the following:

- *Inducing angiogenesis*
- *Resisting cell death (including apoptosis, necroptosis etc.) **
- *Enabling replicative immortality**
- *Sustaining proliferative signalling*
- *Evading growth suppressors*
- *Activation invasion and metastasis*
- *Deregulation of cellular energetics*
- *Avoiding or escape from immune system (destruction)*
- *Tumour-promoting inflammation*
- *Genome instability and mutation*

The two principles, which are marked with an asterisk (*), are all involved in the same principle of creation of immortality of the cell - irrespective of the underlying mechanism.

After establishing the cell lines and the knockdown of PANTR1 with the help of the siRNAs, PANTR1's ability to influence several hallmarks of cancer was investigated.

4.4.1 PANTR1 as a possible key player in angiogenesis/vasculogenesis

The process of formation of new blood vessels from pre-existing vascular structures is called *angiogenesis* whereas creation of completely new vessels without existing structures is called vasculogenesis. All these processes require multiplex and multilevel environment involving sequences of complex remodelling processes such as vasodilation, increase of cellular permeability, endothelial cell proliferation, cell remodelling, migration, survival, differentiation and survival [157,158].

One very interesting fact about PANTR1 is its unique expression in normal as well as cancerous kidney and in brain tissue. Glioma is the also cancerous brain counterpart to RCC and it represents the most frequent primary brain tumour. PANTR1 was found to be highly as well as very differentially expressed in low-grade glioma as well as glioblastoma which represents the most aggressive form among the glioma subtypes. This is considerable as angiogenesis as well as vasculogenesis also play a central role in glioma formation in almost the same relevance as in ccRCC.

In up to 80% of cases of sporadic ccRCC cases, the VHL protein is disturbed. For the formation of ccRCC a mutation is needed in both alleles. Mostly, the function of one allele is lost decades prior to the second. After the 2nd event ccRCC begins to develop [19,157,158]. The underlying pathway involves the cell's mechanisms to respond to hypoxia/hypoxaemia. HIF cannot regularly be detected by the mutated/deactivated VHL protein. The HIF subunits cannot be deactivated by polyubiquitination and so they escape this protection mechanism. This results in a cascade of activation of several genes with involvement of major proteins responsible for angiogenesis such as VEGF, PDGF, EGFR, c-met and cyclin D [8]. In hereditary VHL Syndrome, this mutated VHL protein causes also causes angiogenesis-dependent tumours [159].

As already mentioned, glioma formation is also highly dependent on angiogenesis. Interestingly, glioma cells also undermine the HIF pathway for the creation of new vessels to escape cell control [160]. To cross link this fact to PANTR1, Lang et al. [126] tried to decipher the underlying mechanism and found out that angiogenesis is also induced by exosomes packed with PANTR1 which are created by glioma cells and then delivered to endothelial cells. They also could show the consequent induction of several angiogenic proteins such as

bFGF, VEGFA, bFGFR, and Angio to underline PANTR1's influence on downstream locations of relevant pathways.

Guo et al [125] could provide evidence that PANTR1 is involved in angiogenesis through influence on the notch signalling pathway. The notch signalling pathway is one of the numerous ways for the induction of tumour-created angiogenesis [161]. It is a versatile pathway that originally is needed for correct embryological development of the central nervous system and certain cardiovascular structures [161,162]. In adults, it also plays a role in carcinogenesis [161,162]. Angiogenesis via the notch pathways is mainly maintained by its Dll4 ligand [162]. Another ligand, Dll1 domains plays a less important (or less investigated) role but it is influenced by the POU protein family to which POU3F3 belongs to. It is suggested to maintain the integrity of arteries and to be involved in post-natal arteriogenesis. PANTR1 could possibly interact with POU3F3 to induce the formation of new vessels.

Given all these hints that POU3F3 and PANTR1 might play an important role in vessel formation, several different assays were performed to test this hypothesis. Here, human umbilical vein endothelial cells were used. These are cells derived from the human umbilical cord and therefore are predisposed to deliver clear and unbiased results for vessel-directed investigations with little to no background noise from other cells.
There are no ccRCC- derived blood vessel or endothelial cells available on the market, therefore human umbilical endothelial vein cells were used. They form networks of vessels when cultured in the correct medium - a perfect scenario to test PANTR1's capabilities to influence blood vessel growth. After knockout, a significant effect of PANTR1 on almost all blood vessel characteristics could be observed.

It was not surprising that siRNA2 had a stronger effect as this had been a constant effect throughout all assays. However, it was astonishing how strong this effect was. We analysed the vessel characteristics with the help of a computer software and in most samples, it was not even able to detect relevant blood vessel growth after siRNA2-mediated knockdown.

As a second parameter for angiogenesis, endothelial cell migration was measured. The ability of attracting endothelial cells is one key to build blood vessel and therefore deliver more nutrients and oxygen to the cell. The endothelial cell migration showed that PANTR1 is capable of increasing endothelial cell migration.

For the influence of PANTR1 on downstream targets in the angiogenesis pathways, ccRCC cell lines were used. Finally, PANTR1's ability to influence other major and known players in angiogenesis induction and regulation was tested with the help of a qRT-PCR. Here, surprisingly only 2 markers showed significant lower expression after knockdown of PANTR1 in all 3 ccRCC cell lines: VEGF-A and LAMC-2.
VEGF-A is a known key player in the creation of new blood vessel but also is a target in ccRCC therapy by single- or multikinase inhibitors. It is activated in the cascade following missed HIF deactivation in ccRCC [8,19,21].
LAMC-2 is a gene encoding for a part of the basement membranes of epithelial cells. It is a glycoprotein called laminin-332 which regulates motility and adhesion in these cells [163]. In cholangiocarcinoma, its involvement in angiogenesis was already demonstrated [164]. Furthermore, the increase of LAMC2 has been demonstrated in different kinds of cancers [163–166].

Further correlation analysis based on publicly available data of the GEPIA database, a significant correlation could only be shown for PANTR1 and VEGF-A, but not for PANTR1 and LAMC-2.
This once again confirms the essential role for VEGF-A as a main mediator of angiogenesis in ccRCC. In this context, it is remarkable that the human anti-VEGF-A antibody Bevacizumab could not demonstrate relevant effects in metastatic ccRCC patients regarding CSS and OS [13].
On the first sight, it does not seem to be logical that VEGF-A was chosen as a primary target as HIF is the one common activator of the cascade. This is mainly due to the fact that HIF as a DNA- binding transcription factor was long-time regarded as an unsuitable therapeutic target.
Then, PT-2385 and later PT-2977 (also known as MK-6482 or now called Belzutifan) were invented [167]. They represent a group of small molecules that inhibits the heterodimerization of HIF-2α with HIF-1β. PT2385 was tested in phase I trials (endpoint: maximum tolerated dose) in heavily pre-treated ccRCC and glioma patients resulting in 14% overall response rate (ORR) and 66% of disease control rate. MK-6482 could achieve an ORR of 28% in a comparable patient cohort. Both substances are now being tested alone or in combination with other agents (e.g., Cabozantinib (clinical trial identifier NCT03634540), Lenvatinib/Pembrolizumab (clinical trial identifier: NCT04736706) and others in phase I, II and III trials [168].

Hence, angiogenesis does play an important role and is being targeted constantly. HIF-directed agents have not reached phase III trials yet and VEGF-A direct agent Bevacizumab is not recommended anymore in mRCC in 2021 – at least in Europe. However, it remains a last-line therapy option mostly for countries with lower expenses for health care [8].

One could then conclude that it is far more efficient to aim for multiple targets at the same time in the downstream pathways after HIF/VEGF activation that to aim for their common activator.
This substance class that includes all single- and multikinase inhibitors is called tyrosine kinase inhibitors (TKI). Several downstream targets to VEGF-A are tyrosine kinase, which are differentially inhibited by these substances. They later replaced Bevacizumab, which resulted in longer CSS and OS for mRCC patients. Currently, TKI are used as single substance or in combination with immunotherapeutic agents (Pembrolizumab, Nivolumab, Ipilimumab) to treat metastatic RCC [8].

4.4.2 PANTR1 influences cellular growth

The strong role of PANTR1 in proliferation as a further hallmark of cancer has been established by numerous different experiments in HCC [124], glioma [125,126], eSCC [123,127,128], nSCLC [136], as well as in breast [130], gastric [132], and cervical cancer [134].

A significant hallmark of cancer is the sustainment of proliferative signalling/proliferation. Countless factors like epithelial-to-mesenchymal transition, hypoxia, cell cycle proteins, stem cell differentiation and many more play an important role that influences the cell's ability to proliferate. Proliferative signalling is a very complex topic that is far from fully been understood. It involves numerous pathways just as WnT/Beta-Catenin, PiK3/AKT/mTOR, Notch, Insulin-like Growth Factor, nF-KappaB or Hedgehog – just to list a few [169].

Whether PANTR1 is involved in proliferative signalling in ccRCC was tested with two assays: A WST-1 proliferation assay and a qRT-PCR for a panel of different proliferative genes. Throughout all ccRCC cell lines, a significant effect of PANTR1 on the viability and proliferative effect could be shown. However, PCR for several genes linked to proliferation could not reveal a single candidate that was differentially expressed in all cell lines. E2F,

MCM2, CDK6, CCNB1, MKI67 and MDM2 were differentially expressed at a significant level by both siRNAs but the effect remained limited to one or two of the ccRCC cell lines. Proliferative signalling is diverse, so are PANTR1's effects as we know so far. For HCC, glioma, eSCC, breast and gastric cancer no further information on eventual influence on established pathways (apart from cell viability and proliferation assays) is available.

In cervical [134] and nSCL [136] cancer, regulation/influence of other ncRNAs by PANTR1 was observed. In cervical cancer, PANTR1 correlates inversely with the expression of miRNA127-5 and they may even share a common binding site. MiRNA127 is a known tumour suppressor with prognostic relevance in several types of cancer [134,170–173]. In thyroid cancer circular RNA circ_0103552 is known to sponge miRNA 127 [173]. In cervical cancer, there are hints for the same mechanism due to a common binding site as mentioned above [134].
A similar effect might occur between PANTR1 and miRNA30d-5p in nSCLC [136]. MiRNA 30d is also a known tumour suppressor in different kinds of cancer [136,174]. The authors conclude that PANTR1 is an upstream regulator of miRNA30 and consequently cyclin E2. It is involved in cell cycle regulation in the G1-S1 phase.

Influence on proliferation of PANTR1 may be diverse, but not enough research has yet been made to finally depict its relevance.

4.4.3 PANTR1 influences cell death/apoptosis

PANTR1 has been demonstrated to influence apoptosis in breast [130] and colorectal [135] cancer which represent two of the three most common cancer types in humans.

For many years, apoptosis and necrosis were believed to be the 2-sided face of cell death in eukaryote cells. Recent intensive research has revealed further subtypes and cell death can now be categorized differently and also more precisely. Classification of the different modes of cell death now ranges from *apoptosis* (still programmed cell death without collateral damage), over *pyroptosis* (programmed cell death with collateral damage) and *autophagy* (mechanism to protect and destroy stressed cells) to *necrosis* (uncontrolled cell death with collateral damage), *necroptosis* (regulated necrotic cell death) and *oncosis* (switching mechanism between apoptosis, autophagy and necrosis) [148].

Apoptosis is the form of self-controlled death of the cell with organised fragmentation following morphological changes along one of the two predefined pathways: An intrinsic (receptor-mediated) and an extrinsic pathway caused by external cytotoxic impulse such as heat or massive change in pH. Whatever it may have initiated, the process is executed by endoproteases, the so-called c*aspases*, which are classified into initiator and effector caspases. Caspases 3 and 7 are two of the three main effector caspases which, upon activation, knock off the process of “DNA destruction, denaturation of nuclear proteins and the cytoskeleton, the expression of ligands for phagocytic cells and the formation of apoptotic bodies“ [148,149].

In our assay, we tested for the presence of caspases 3 and 7 of in three different ccRCC cell lines with and without knockdown of PANTR1. Throughout all 3 cell lines and with both siRNAs, significant downregulation of the expression could be demonstrated. This is a clear hint that PANTR1 is involved in deactivation of the intrinsic pathways in ccRCC cell. These results and consequently PANTR1’s role in apoptosis in ccRCC was confirmed by a Western Blot of PARP/cleaved PARP [1].

In colorectal cancer cell lines, Shan et al. could also show that PANTR1 influences autophagy signalling apart from influencing the process of apoptosis [135]. Yang et al [130] could confirm the involvement of PANTR1 in breast cancer cells, but no correlation between Caspase 9 und PANTR1 levels could be found in healthy tissue. They concluded that there is no direct link between the two and there must exists any form of mediator to communicate. Whether this can be concluded just from one test in one cancer subtype remains doubtful.

Unfortunately, neither these two authors nor the author of this dissertation can provide any kind of solid background information how PANTR1 influences apoptosis.

4.5 Is there a role for PANTR1 in other subtypes of RCC?

Whether there is a role for PANTR1 in other subtypes of RCC is speculative. Hypothesis only can be postulated as no specific scientific research has yet been performed.

However, some hints are given by RNA sequencing data from the NCBI gene database. It was already demonstrated that PANTR1 is expressed in adult and foetal renal tissue. When regarding PANTR1 expression in different subtypes of RCC, ccRCC is not the only one with relevant expression. As already explained, two other major subtypes of RCC, namely chRCC and pRCC, account for approximately 15% of all cases of RCC. Their cancer pathways completely differ from ccRCC's and even within these 2 subtypes, different sub-subtypes exist.
The clinically most relevant ones are the 2 main subtypes of pRCC, pRCC type I and pRCC type II. In pRCC type I, the MET pathway is frequently altered – a pathways that also plays a role in ccRCC. This is underlined by the fact that a TKI influencing the MET pathway is currently used in during the treatment of metastatic ccRCC [175]. Currently, only Cabozantinib is licensed in Europe for this treatment option while several others are currently being tested in phase II trials (e.g. Crizotinib, Savolitinib, Foretinib) [20]. In pRCC type II, similar, but also completely different genetic alterations compared to ccRCC could be demonstrated. Similar changes include alterations of the SETD2, BAP1 and PBRM1 gene while NF2, TERT and NRF2-ARE as examples for differentially expressed genes can also be altered [175].

Regarding a possible role and oncogenic function of PANTR1 in these two subtypes of RCC, available data is scarce. Data from the NCBI database suggests a role in pRCC. In pRCC, PANTR1 was 50% overexpressed in comparison to normal renal tissue. This is less than in ccRCC, where PANTR1 is 100% overexpressed in comparison to normal kidney tissue, but still is considerable.
Furthermore, interpretation in context of the biologically different subtypes of pRCC is not facilitated by unstratified data resulting in one median pRCC RNA expression. But regarding possible crosslinks between ccRCC and both subtypes of pRCC, PANTR1 may also play an (oncogenic?) role in pRCC.

On the contrary, expression in chRCC is nearly 4-fold lower than in normal kidney tissue. How this fact can be interpreted is not clear at the moment. Currently, there are no evidence

whether under-expression of PANTR1 does play a role in carcinogenesis as in all investigated types of cancer, PANTR1 was overexpressed and displayed pure oncogenic functions.

In conclusion, there is simply not enough data to give an evidence-based opinion on a possible role for PANTR1 in other subtypes of RCC. Further research is needed to provide reliable data to draw further conclusions.

4.6 Further research opportunities

As always in experimental/translational research, further research could have been conducted.

Firstly, the role of the protein-coding gene POU3F3, its mRNA expression and the protein POU3F3 itself could be a target for further investigation. The interplay between PANTR1 and POU3F3 has been investigated for glioma [125] but no data exists for ccRCC. Furthermore, the role of POU3F3 in different types of cancer as well as its possible role as an epigenetic regulator could be of scientific interest. Additionally, POU3F3's role in ccRCC as well as in other subtypes of RCC, especially chRCC and pRCC type I and type II, remains completely unclear. An inverse correlation between PANTR1 and POU3F3 mRNA has been demonstrated for HCC [124] and glioma [125] with likewise inverse correlation between PANTR1 expression and POU3F3 gene expression in eSCC [123]. However, in eSCC PANTR1 expression induced POU3F3 mRNA expression [123].

Cellular localisation could also be of interest as there is only one publication dealing with this issue in general. Li et al [123] found PANTR1 to be located in the cytoplasm as well as in the nucleus in glioma cells, but no information exists for ccRCC/RCC. In this context, stimulation/overexpression of PANTR1 could be tested.
Further analysis could include all other hallmarks of cancer.

Secondly, the role of PANTR1 in other subtypes of RCC is completely unclear. As other subtypes of RCC share some but definitely not all molecular features of ccRCC, it remains speculative whether PANTR1 might there play a role. Currently, we know that therapeutics aiming for VEGF and its downstream targets do not have a relevant impact on OS of mRCC patients, who suffer from these subtypes. The 20-year CSS after surgery for these two

subtypes is well above 80% compared to 50% in ccRCC regardless of the initial tumour stage. Having in mind this fact as well as the far lower amount of cases, it is doubtful whether further investigation will ever be conducted.

Thirdly, the different pathways PANTR1 interacts with, remain mostly speculative. Investigations which aim to decipher the interaction between PANTR1 and different other proteins could be conducted (e.g. RNA immunoprecipitation assay).
Furthermore, PANTR1's role as an epigenetic regulator has only been investigated once before in eSCC [123]. Therefore, verification of these results in eSCC and in other types of cancer would be of interest. Additionally, interaction with all the currently clinically relevant pathways ranging from HIF over VEGF to MET, mTOR, KDR and AXL etc. could be investigated.

Fourthly, lncRNA are known to cause resistance to established therapies. For several different lncRNAs, resistance mechanism to overcome treatment with Sorafenib, Sunitinib, 5-FU and cisplatin in RCC cells have been demonstrated. [101,102]. Changes on the behaviour of RCC cells after knockdown or overexpression of PANTR1 AND after treatment with different substances (e.g. TKIs, checkpoint- and mTOR-inhibitors) could be investigated. Further analysis could also compare PANTR1's expression before and after treatment with these substances.

Fifthly, further testing could also include other ccRCC cell lines. Choosing the correct cell line represents a crucial step to obtain reliable results in *in-vitro* cancer research. Numerous cancer cell lines provided by countless producers worldwide at different levels of quality exist. RCC research is principally enforced on cell lines extracted and created from tumours that were originally diagnosed as ccRCC [176]. This is the logical conclusion as ccRCC represents two third of all and 85% of all metastatic cases. Furthermore, clinical research in the hunt for more powerful therapeutics is almost exclusively realised on patients with advanced or metastatic ccRCC. We therefore aimed for three different ccRCC cell lines displaying the typical feature of VHL mutation as the representative example. For more than a decade and prior to the introduction of novel immunotherapy (other than cytokines), it has been the main target for first- and second-line therapeutics such as TKI and mTOR inhibitors. Other typical characteristics such as AXL, PI3K/AKT or c-met status which also are of clinical interest, have mostly not been characterized in these cell lines [176]. Therefore, cell lines 769-P, RCC-FG and RCC-MF were selected the to show a persistent effect of PANTR1 in

these heterogeneous examples of ccRCC. To perfectly ensure whether to have chosen the correct cell lines, complex molecular characterization of these obtained cell lines would have been needed.

Sixthly, the role of different siRNAs could be investigated. Knockdown efficiency was tested in the beginning with the help of qRT-PCR. PANTR1 expression was proven to be significantly decreased. Expression level were almost the same for both siRNAs. On the contrary, we partly could observe a massive difference in regard to the knockdown effect of both siRNAs in different assays. The most obvious effect could be demonstrated for the tube formation assays investigating angiogenesis. Here, the effect of one of the two siRNAs was so strong in regard to several parameters that it could not even be measured by the analysis software. Both siRNAs were purchased from the same company and we could not find a solution for this disbalance in their data sheets. Further siRNAs by other companies exist, so possible further or different effect could also be possible and could be worth being investigated.

Seventhly, additional analysis of ccRCC cell lines obtained from metastasis could be performed to investigated the expression patterns of PANTR1 and their behaviour in regard to the hallmarks of cancer. Finally, they could be compared to our results in ccRCC cell lines obtained from primary tumours. It is completely unclear whether PANTR1 would play another (more/less important role) in RCC metastasis.

4.7 Conclusion

In this thesis, lncRNA PANTR1 could be identified as a player in ccRCC carcinogenesis.

Based on our findings, PANTR1 seems to influence proliferation as well as cellular growth in cancer cell lines also through the regulation of apoptosis. Furthermore, PANTR1 seems to be involved in angiogenesis. This process seems further to involve VEGF-A (and LAMC2), both previously recognized regulators of angiogenesis.

Further studies are warranted to identify the underlying molecular mechanisms and to identify and test possible drugs which target PANTR1.

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