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The role of Grainyhead-like 3 transcription factor (GRHL3) in urothelial bladder cancer

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Abbreviations

AEC	3-Amino-9-Ethylcarbazole
AJCC	TNM staging system of The American Joint Committee on Cancer
APS	Ammonium persulfate
BCA	Bicinchoninic acid
BCC	Basal cell cancer
BOM	Brother of mammalian grainyhead
bp	Base pairs
BPE	Bovine pituitary extract
BSA	Bistrimethylsilylacetamide
cDNA	Complementary DNA
CIM	Cell invasion and migration
CIS	Carcinoma in situ
CO2	Carbon dioxide
CRC	Colorectal cancer
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DMSO	Dimethyl sulfoxide
E. coli	Escherichia coli
EDTA	Ethylene diamine tetra-acetic acid
EGF	Endothelial growth factor
EGTA	Ethylene glycol-bis tetra-acetic acid

FBS	Fetal bovine serum
FCS	Fetal calf serum
FGFR3	Fibroblast growth factor receptor 3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
gGFP	Guide green fluorescent protein
GRHL1	Grainyhead-like protein 1 homolog (Homo sapiens)
GRHL2	Grainyhead-like protein 2 homolog (Homo sapiens)
GRHL3	Grainyhead-like protein 3 homolog (Homo sapiens)
GRHL3	Grainyhead-like transcription factor 3 (Homo sapiens)
GSMT1	Glutathione S-transferase mu 1
HCl	Hydrochloric acid
HE	Haemalm eosin
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HNPCC	Hereditary nonpolyposis colorectal cancer
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IHC	Immunohistochemistry
KB	Kilo bases
kDa	Kilo Dalton
MAPK	Mitogen-activated protein kinase
MgCl2	Magnesium chloride
MGR	Mammalian grainyhead
MIBC	Muscle invasive bladder cancer
miRNA	MicroRNA

mRNA	Messenger RNA
MTOR	Mechanistic target of rapamycin kinase
NMIBC	Non-muscle invasive bladder cancer
NP-40	Nonyl phenoxypolyethoxylethanol
O2	Oxygen
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PIK3	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and tensin homolog
P/S	Penicillin Streptomycin
q-RT-PCR	Quantitative real time polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNase	Ribonuclease
RPM	Rounds per minute
RPMI	Roswell Park Memorial Institute
RTCA DP	Real Time Cell Analyzer – Dual Purpose
SCC	Squamous cell cancer
SDS	Sodium dodecyl sulfate
Ser	Serin
SFM	Serum free medium
shRNA	Small hairpin RNA
SLC14A1	Solute carrier family 14 member 1
SOC	Super Optimal broth with Catabolite repression

SOM	Sister of mammalian grainyhead
SP1	Sp1 transcription factor
TBE	Tetrabromoethane
TBP	TATA-box binding protein
TBST	Tris-buffered saline tween
TEER	Transepithelial electrical resistance impedance
TEMED	Tetramethylethylenediamine
TGase1	Transglutaminase 1
Thr	Threonine
TGX	Tris glycine extended
UP	Uroplakin
UPK	Uroplakin
WB	Western blot

1. Introduction

1.1 Bladder Cancer

1.1.1 Epidemiology

Cancer diseases are a very common cause of death and urinary bladder cancers are one of them. In 2013 the age-standardized incidence for bladder cancer was 9.1 per 100,000 for women and 36.1 per 100,000 for men in Germany with a median age of diagnosis of 73.4 in women and 71.9 in men. It accounted for an age-standardized death rate of 1.9 per 100,000 in women and 6.2 per 100.000 in men. [7] In the global scheme of things bladder cancer is the ninth most prevalent malignant disease and the thirteenth most prevalent cause of death due to cancer. [72] When diagnosed in one fourth of male and one third of female patients the tumour is already muscle invasive. [7] In 90 % of the cases bladder cancer originates from the urothelium. Less common types are squamous cell carcinoma, small-cell carcinoma and adenocarcinoma. [72] Squamous cell carcinoma is a common histological type in countries with schistosomiasis, for example in Egypt. [11, 25]

1.1.2 Risk factors

The most important risk factor for bladder cancer is smoking. [7, 12, 72] But exposure to other carcinogens can lead to a higher incidence of bladder cancer and a higher bladder cancer specific mortality. Chemical process, rubber and dye workers show a significant increase in bladder cancer incidence. Other occupational groups like waiters, healthcare workers and electrical workers also suffer from an increased risk of bladder cancer. The greatest bladder cancer specific mortality is seen in electrical, chemical process and transport workers. [20, 72] Responsible chemicals are amongst others aromatic amines. [2, 67, 70] The drug cyclophosphamide also increases the risk of bladder cancer. [50, 57, 77]

Genetic predisposition was reported to play a critical role in cancer development in some patients. It was shown, that patient with hereditary nonpolyposis colorectal cancer (HNPCC), also known as Lynch syndrome, have a greater risk of developing bladder cancer, especially if they have mutations in *MSH2*. [23, 71, 79, 84] Two other examples are mutations in N-Acetyltransferase with slower acetylation or a *GSTM1* deletion. [3, 29, 63] Schistosomiasis is a risk factor for squamous cell carcinoma of the bladder. [11, 25, 66]

1.1.3 Pathogenesis

Since the pathogenesis of bladder cancer differs dependent on the staging, I will at first introduce the TNM staging system of The American Joint Committee on Cancer (AJCC). The main categories are T (local tissue invasion of the tumour), N (regional lymph node metastasis) and M (distant metastasis). Table 1 gives detailed information on the TNM staging system. [34, 59]

Table 1 TNM Staging System of The American Joint Committee on Cancer (AJCC) of bladder cancer form 2017 [59]

Classification	Tumour extent
T	Primary tumour
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ
T1	Tumour invades subepithelial connective tissue
T2	Tumour invades muscularis propria
T2a	Tumour invades superficial muscularis propria (inner half)
T2a	Tumour invades deep muscularis propria (outer half)
T3	Tumour invades perivesical tissue
T3a	Microscopically
T3b	Macroscopically (extravesical mass)
T4	Tumour invades any of the following organs
T4a	Prostatic stroma, seminal vesicles, uterus, vagina
T4b	Pelvic wall, abdominal wall
N	Regional lymph nodes
Nx	Lymph nodes cannot be assessed
N0	No lymph node metastasis
N1	Single regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac or presacral lymph node)
N2	Multiple regional lymph node metastasis in the true pelvis
N3	Lymph node metastasis to the common iliac lymph nodes
M	Distant metastasis
M0	No distant metastasis
M1	Distant metastasis

Bladder cancer can be classified into two categories. One category regards the papillary non-muscle invasive bladder cancer (NMIBC), which is limited to the mucosa (Ta, Tis) or to the submucosa (T1). [24] The other category is called

muscle invasive bladder cancer (MIBC). MIBC has T2 or more in the TNM staging system. Approximately 75 % of patients have NMIBC and 25 % have MIBC or metastases at the time of diagnosis. [6, 72] Tumorigenesis of bladder cancer is often associated with deletion of chromosome 9 in both categories. [19, 31, 64, 80] Beyond that there are two ways of further tumorigenesis known. One way leads to NMIBC and the other leads to MIBC. [42]

NMIBC usually arises from hyperplasia. [42] Studies showed that in NMIBC *FGFR3*, a receptor tyrosine kinase, or proteins belonging to *RAS* family frequently acquire activating mutations but they seem to be mutually exclusive. Both mutations activate the MAPK pathway and stimulate cell proliferation. *FGFR3* undergoes more mutations in Ta tumours than in T1 tumours and more often in low grade tumours than in high grade tumours. [16, 32, 44, 51, 61, 81, 82] *PIK3CA* also is often mutated, in many cases together with *FGFR3*. [53, 56]

MIBC usually arises from dysplasia via Carcinoma in situ (TIS). [42] It usually presents mutations in *P53* and *RB1* leading to CIS and invasive tumours. [24, 31, 48, 69] Mutations in the *PIK3*/*MTOR* pathway or its upstream effectors, leading to inactivation of the pathway and thereby to proliferation, are also playing an essential role. In this pathway *AKT* plays an important role, but in cancer it is usually not mutated. This pathway also is inhibited by *PTEN* which is often mutated in bladder cancer. [5, 15, 45, 49, 68] Clusters of mRNA expression allow the classification into five different subtypes: luminal-papillary (35%), basal-squamous (35%), luminal-infiltrated (19%), luminal (6%) and neuronal (5%). [69]

These two different ways of tumorigenesis lead to a broad spectrum of different diseases. The WHO classification of urothelial bladder cancer from 2016 has divided invasive and non-invasive urothelial bladder cancers in different sub-types. (Table 2). After [41]

Table 2 WHO classification of urothelial tumours of bladder from 2016 after [41]

Infiltrating urothelial carcinoma	Non-invasive urothelial neoplasms
Nested, including large nested	Urothelial carcinoma in situ
Microcystic	Non-invasive papillary urothelial carcinoma, low-grade
Micropapillary	Non-invasive papillary urothelial carcinoma, high-grade
Lymphoepithelioma-like	Papillary urothelial neoplasm of low malignant potential
Plasmacytoid/signet ring cell/diffuse	Urothelial papilloma
Sarcomatoid	Inverted urothelial papilloma
Giant cell	Urothelial proliferation of uncertain malignant potential
Poorly differentiated	Urothelial dysplasia
Lipid-rich	
Clear cell	

In normal bladders five related proteins, the uroplakins (*UP*) Ia, Ib, II, IIIa and IIIb, are expressed in the apical cells. They form the urothelial plaques and are conserved through most mammalian species. [55, 88, 91] Besides the urothelium *UPIb* is expressed in the respiratory tract, for example in squamous metaplasia, while *UPIII* is expressed in prostate. *UPIa* and *UPII* are only expressed in the urothelium. In urothelial bladder cancers no uroplakin is expressed in all primary tumours or metastases. In 80% of primary tumours and 70% of lymph node metastases at least one uroplakin is detectable. Several cases of urothelial bladder cancer were found with expression of Uroplakins in metastases, although the primary tumour lacked expression. So the expression of both UPIa and UPII is highly specific to urothelium, but the differentiation-restricted expression may be lost during tumorigenesis. [65] These results were confirmed by other studies: although the uroplakins are regarded as markers for urothelial cell differentiation, several studies showed that these proteins are expressed in more than half of invasive and metastatic cancers and nearly 90 percent of non-invasive cancers.

[39, 62, 65] In contrast to normal urothelium the UPs are localized aberrantly in cancer tissue, meaning that the UPs are not only expressed at the apical surface. Correlations between *UP* expression and disease recurrence, progression and mortality were investigated for non-muscle-invasive and muscle-invasive cancers. In non-muscle-invasive cancers was no correlation found, but absent *UP* expression and “advanced pathologic stage, lymph node metastases, disease recurrence, and bladder cancer–specific mortality” correlated in muscle-invasive cancers. [39]

1.2 Grainyhead like transcription factor 3 (*GRHL3*)

1.2.1 Family of *GRHL3*

The members of the family of Grainyhead (*GRH*) transcription factors are conserved in several species and the genes have their roots before the divergence of animals and fungi. [78] The three family members are Grainyhead-like transcription factor 1 (*GRHL1*), Grainyhead-like transcription factor 2 (*GRHL2*) and Grainyhead-like transcription factor 3 (*GRHL3*), formerly known as *MGR*, *BOM* and *SOM*. [4, 52, 76] They are capable of homo- and heterodimerization with each other. [76, 87] Also they have a highly conserved DNA-binding domain. [52]

1.2.2 Gene, protein structure and function of GRHL3

GRHL3 is located on chromosome 1p36.11. [35, 36, 52] It has 45,126 bp and 19 exons. [35] These exons arrange to five isoforms by splicing. The resulting protein is called Grainyhead-like protein 3 homolog (GRHL3), also known as Sister of mammalian grainyhead (SOM). Isoform 1 has 626 amino acids and weights 70,345 Da (Q8TE85-1), Isoform 2 has 607 amino acids and weighs 68,353 Da (Q8TE85-2), isoform 3 has 556 amino acids and weighs 62,378 Da (Q8TE85-3), isoform 4 has 509 amino acids and weighs 57,045 Da (Q8TE85-4) and isoform 5 has 602 amino acids and weighs 67,696 Da (Q8TE85-5). [38]

GRHL3 is exclusively expressed in the nucleus and mainly in squamous epithelia. [37, 52] It has the role of a DNA-binding protein and acts as activator and repressor. [52] GRHL3 binds to the binding site 5'-AACCGGTT-3'. GRHL1 and GRHL2 share the identical binding site. The activated genes depend on the different dimers between the three proteins. [10, 75, 86] Furthermore, GRHL3 binding to the DNA depends on the presence of other transcription factors. It cannot activate gens alone. [27] Recently, Jacobs et al. showed that GRHL3 (as well as GRHL1 and GRHL2) do not bind directly to enhancers. But by binding to its binding site, it makes enhancers accessible to repressors and activators. It achieves this via nucleosome displacing. That explains why presence of GRHL3 leads to activation and repression depending on the target gene. [43] DNA methylation plays an important role, too. [27, 33] GRHL3 has a least several hundred targets. [30, 33] Isoform 1 was reported to have 367 target genes and isoform 3 was reported to have 261 target genes. [30] Its functions, especially in differentiation of the urothelium and the skin, are explained below.

In mice the expression of *Grhl3* in the urothelium highly correlates with the expression of the Uroplakins (e.g. *UpII*), mentioned above as differentiation markers, and in mice lacking *Grhl3* these genes are downregulated. *UpII* is a direct target of Grhl3 in murine bladder epithelium. In murine bladder *Grhl3* is mostly expressed in umbrella cells, whereas in the ureter it is expressed in the intermediate and superficial layers. Loss of *Grhl3* is associated with enlarged bladders in mouse embryos. Additionally, the umbrella cells in these mice show changes in shape during development, indicating the necessity of *Grhl3* during urothelial development. The umbrella cells are smaller, have microvilli instead of ridges and have less vesicles transporting Uroplakins. Moreover the barrier function of the urothelium is compromised in these mice and tight-junctions are impaired. But *Grhl3* seems not to have an influence on the proliferation of urothelial cells. [91] In normal human urothelial cells a knock-down of *ELF3* led to a decreased expression of *GRHL3* and *UPIIIa* and a impaired barrier forming and repair in transepithelial electrical resistance studies. [9]

In the human keratinocyte cell line HaCaT that lost *GRHL3* cells grew less dense and the colonies were smaller. [26] This was mediated by upregulation of the thymus and activation-mediated chemokine (TARC). [26] Upregulation of TARC

following the *GRHL3* knock-down was reported to increase blood clotting in an in-vitro model. [26] In mice Grhl3 regulates wound healing, cellular polarity and actin polymerisation in keratinocytes via its target gen *Rhogef19*. [13]

Mice lacking *Grhl3* show a hyperproliferation of the epidermis [21] and a pro-tumorigenic state of dedifferentiated cells. In addition, the skin of *Grhl3* deficient mice showed a pro-inflammatory respectively pro-proliferative micro-environment. [26] Furthermore, mouse embryos lacking *Grhl3* showed a defective skin barrier and had a limited ability of wound healing. [75, 90] Mice die from dehydration because of the defective skin barrier when they lack *Grhl3* in utero. When they lose the gen postnatal the skin barrier is intact and they show the changes mentioned above. [14] But the cornified layer itself is not fragile. Permeability originates from a different expression of tight junction proteins and a different lipid composition: phospholipids are increased and fatty acids are decreased. [90] Amongst others, this is mediated by reduced levels of the tight junction protein cross-linking enzyme Transglutaminase 1 (TGase1), whose recruitment depends on Grhl3, in embryos. [33, 75] Eyelid-closure fails in mice embryos without *Grhl3*. Grhl3 is a upstream regulator of *Tgfα*, *Egfr* and *Erk*, which have an important role in eyelid-closure, in epidermal keratinocytes of these animals. [89] Impaired differentiation of the epidermis in *Grhl3* knock-down mice can be objectified by the altered expression of differentiation markers *involucrin*, *loricrin* and *filaggrin*. [90] So all in all, *Grhl3* has an important role in skin differentiation.

In human endothelial cells the isoforms 1 and 3 have different effects in vitro. The isoform 1 increases the migration capacity and the isoform 3 decreases it. Isoform 1 inhibits apoptosis, whereas isoform 3 has no effect. Additionally it was shown that isoform 2 has an anti-apoptotic effect and induces migration. [30] These effects were mediated by expression and downregulation of *ENOS*, *AKT2* and *Max interactor 1*, that are important for migration and apoptosis. [30, 58]

Both increase and decrease of Grhl3 levels lead to spina bifida in mice, because both the loss and the overexpression lead to a larger posterior neuropore length. That indicates the important role of *Grhl3* in neural tube closure. [17] The same result was shown in zebrafish studies. Studies also showed that human foetuses with neural tube defects have a hypomethylation in the *GRHL3* gene. [74] Additionally it was shown in mice that for correct neurulation *Grhl3* expression is

not only required in neuroepithelium but also in the surface ectoderm and in the hindgut. [22]

1.2.3 GRHL3 in Cancer

The function of *GRHL3* has been investigated for several cancers. Yet, *GRHL3* has still not a clear role as a oncogene or a tumour suppressor. In fact, its role depends on the disease in which it is investigated. I will further explain this in detail below.

A tumour suppressor effect was shown in the skin. The lack of GRHL3 leads to an increasing risk of developing squamous cell cancer (SCC). [8, 21] In SCC and basal cell cancer (BCC), both skin cancers, GRHL3 and phosphatase and tensin homologue (PTEN) levels are reduced compared to healthy epidermis. There are no de novo single nucleotide mutations in *GRHL3* compared to healthy tissue of the patients, but patients show increased frequencies of rare polymorphisms. [21, 47] Amongst others, *PTEN* is a direct target of GRHL3 in the human keratinocyte cell line HaCaT. GRHL3 has a binding site in the *PTEN* promoter. PTEN affects the PI3K/AKT pathway and the MAPK/ERK pathway. Both *PTEN* and *GRHL3* are targets of miRNA-21. [21] One study showed that in breast cancer GRHL3 levels are high in early and low in advanced stages, indicating a correlation between GRHL3 levels and prognosis. [40] One possible explanation for these results is the regulatory effect of GRHL3 on *E-Cadherin*. It was shown that murine *E-Cadherin* is a target of Grhl3 and in murine mammary tissue the expression of *Grhl3* and *E-Cadherin* correlate. In human breast cancer cell lines Cal51 and MDA-MB-231 *GRHL3* and *E-Cadherin* expression correlate. [1]

On the other side, another study showed inverse levels of E-cadherin and GRHL3 the breast cancer cell lines MCF-7, A-431 and MDA-MB-231. GRHL3 also increases invasion and migration in these cell lines. [92] These results support the oncogenic role in cancer. In patients with diffuse large B cell lymphoma GRHL3 is an independent prognostic factor. Patients with GRHL3 positive tumours have a worse survival. [54] Studies showed that *GRHL3* expression is elevated in colorectal cancer (CRC). Knock-down in CRC cell lines DLD1 and HT29 reduces

the colony forming efficacy, migration and proliferation potential of the cells and leads to cell cycle arrest and apoptosis. [83] In addition, GRHL3 is upregulated in advanced and pre-treated stages of small cell lung cancer and adenocarcinoma. [73] In tumours *GRHL3* is a target of TNFα. As described above, GRHL3 induces endothelial cell migration. This pathway could explain how TNFα and GRHL3 have a pro-tumorigenic role via vascularization in tumours. [28]

1.3 Aim of Study

Although a role of *GRHL3* is indicated in different cancer entities, a contribution of *GRHL3* was not investigated in urothelial bladder cancer. Based on the expression of *GRHL3* in bladder epithelium, its proposed role in cell differentiation and development of epithelia and its role in different cancer entities, we aimed to investigate the impact of *GRHL3* on tumourigenesis of urothelial bladder cancer. For this purpose, *GRHL3* was ectopically expressed in the T24 bladder cancer cell line, that lacks the expression of this gene, and conversely knocked down in RT4 and HT1197 cell lines with high respectively moderate endogenous expression. Using this model, we investigated how *GRHL3* changes different aspects of the tumour. Migration was investigated with the wound healing assay. To see how *GRHL3* alters invasion, a porcine bladder organoid model and continuous monitoring of cell impedance were used. Then we looked into the effect of *GRHL3* on differentiation markers, signal transduction proteins und cell cycle protein.

2. Material and Methods

2.1 Materials

2.1.1 Chemicals

All used chemicals are listed in table 3.

Table 3: Chemicals and their provider and headquarter.

Chemical	**Provider and headquarter**
10X FastDigest buffer 10X smart buffer?	ThermoFisher Scientific, Waltham (USA)
10X PBS pH = 7.4	Invitrogen, ThermoFisher Scientific, Waltham (USA)
2-mercaptoethanol	Sigma-Aldrich, St. Louis (USA)
2-propanol	VWR chemicals, Radnor (USA)
4X Laemmli sample buffer	Bio-Rad Laboratories Inc., Hercules (USA)
6X DNA loading dye	ThermoFisher Scientific, Waltham (USA)
Accutase® solution	Pan Biotech, Aidenbach (GER)
AceGlow-Solution A chemiluminescence substrate	VWR International, Radnor (USA)
AceGlow-Solution B chemiluminescence substrate	VWR International, Radnor (USA)
Acetic acid glacial 100 %	MerckMillipor, Darmstadt (GER)
AEC substrate chromogen	Agilent technologies, Santa Clara (USA)
Albumin bovine fraction V pH = 7.0	Serva, Heidelberg (GER)
Ammonium peroxide sulphate (APS)	Bio-Rad Laboratories Inc., Hercules (USA)
Ampicillin	Sigma-Aldrich, St. Louis (USA)
Bicinchoninic acid solution	Sigma-Aldrich, St. Louis (USA)
Blasticidin S HCl	Gibco life technologies, ThermoFisher Scientific, Waltham (USA)
Bovine pituitary extract	Gibco life technologies,

	ThermoFisher Scientific, Waltham (USA)
Cell lysis buffer 10X	Sigma, St. Louis (USA)
Concentrated (10X) antigen retrieval Citra plus solution	BioGenex, San Ramon (USA)
Target Retrieval Solution (10x) pH = 7	Agilent technologies, Santa Clara (USA)
Copper (II) sulphate solution	Sigma-Aldrich, St. Louis (USA)
Corning® Matrigel® Matrix	Corning, New York (USA)
ddH_2O	University Ulm, Ulm (GER)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis (USA)
Dispase II	Sigma-Aldrich, St. Louis (USA)
DL-Dithiothreitol (DTT) solution (1 M)	Sigma-Aldrich, St. Louis (USA)
DNase free water	Qiagen, Hilden (GER)
DNAZap solution 1	Invitrogen, ThermoFisher Scientific, Waltham (USA)
DNAZap solution 2	Invitrogen, ThermoFisher Scientific, Waltham (USA)
dNTP's	Promega Corporation, Madison (USA)
Dulbecco's modified eagle's medium – high glucose	Sigma-Aldrich, St. Louis (USA)
Dulbecco's phosphate buffered saline	Gibco life technologies, ThermoFisher Scientific, Waltham (USA)
Endothelial growth factor (EGF human recombinant)	Gibco life technologies, ThermoFisher Scientific, Waltham (USA)
Entellan® new	MerckMillipor, Darmstadt (GER)
Eosin solution	Waldeck, Münster (GER)
Ethanol	Honeywell, Morris Plains (USA)
Ethylene diamine tetra-acetic acid [EDTA (Versen) 1 % (w/v) in PBS w/o Ca^{2+}]	Biochrom, Berlin (GER)
Ethylene glycol tetra-acetic acid (EGTA)	Sigma-Aldrich, St. Louis (USA)
10X FastAP buffer	ThermoFisher Scientific, Waltham (USA)

FastDigest™ BsmBI (Esp3I)	ThermoFisher Scientific, Waltham (USA)
Fetal bovine serum	Sigma-Aldrich, St. Louis (USA)
Formaldehyde solution 37 %	CarlRoth, Karlsruhe (GER)
Gelatine 1 %	Pan Biotech, Aidenbach (GER)
GeneRuler™ 1 kb Plus DNA ladder	ThermoFisher Scientific, Waltham (USA
GeneRuler™ 50 bp Plus DNA ladder	ThermoFisher Scientific, Waltham (USA
GlutaMAX -I (100X)	Gibco life technologies, ThermoFisher Scientific, Waltham (USA)
Glycerol 89 %	MerckMillipor, Darmstadt (GER)
Glycine	Labochem international, Einhausen (GER)
GoTaq® G2 flexi DNA polymerase	Promega Corporation, Madison (USA)
Hepes buffer (1M)	Biochrom, Berlin (GER)
Hydrochloric acid 30 %	AppliChem, Darmstadt (GER)
Hydrochloric acid solution 100 %	J.T. Baker, ThermoFisher Scientific, Waltham (USA)
Hydrocortisone	Sigma-Aldrich, St. Louis (USA)
Iron (II) sulphate heptahydrate	Sigma-Aldrich, St. Louis (USA)
iTaq™ Universal SYBR®Green supermix	Bio-Rad Laboratories Inc., Hercules (USA)
Keratinocyte-SFM 1X	Gibco life technologies, ThermoFisher Scientific, Waltham (USA)
L-ascorbic acid	Sigma-Aldrich, St. Louis (USA)
LB Broth (Miller)	Sigma-Aldrich, St. Louis (USA)
Mayer's hemalum solution	MerckMillipor, Darmstadt (GER)
Methanol	Honeywell, Morris Plains (USA)
$MgCl_2$ 25 mM	Promega Corporation, Madison (USA)
Non-essential amino acids (100x)	Biochrom, Berlin (GER)
NP-40 100 ml	Abcam, Cambridge (UK)
Nuclease free water	Promega Corporation, Madison (USA)
One shot® Stbl3™	Invitrogen, ThermoFisher Scientific, Waltham (USA)
Pageruler plus prestained protein	ThermoFisher Scientific, Waltham (USA)

ladder	
Paraplast Plus, tissue embedding medium	McCormick Scientific, Saint Louis (USA)
Penicillin-streptomycin	Pan Biotech, Aidenbach (GER)
PeqGreen DNA/RNA dye	PEQLAB Biotechnologie GmbH, Erlangen (GER)
Peroxidase blocking solution	Agilent technologies, Santa Clara (USA)
Pierce™ Protein-Free T20 (PBS) Blocking Buffer	ThermoFisher Scientific, Waltham (USA)
PhosphoSTOP EASYpack	Roche, Rotkreuz (GER)
Polybrene	Sigma-Aldrich, St. Louis (USA)
Polyethylenimine	Polyscience, City of Brotherly Love (USA)
Potassium chloride	MerckMillipor, Darmstadt (GER)
Proteaseinhibitor cOmplete tablets EASYpack	Roche, Rotkreuz (GER)
Protein standard	Sigma-Aldrich, St. Louis (USA)
Puromycin	Gibco life technologies, ThermoFisher Scientific, Waltham (USA)
RIPA buffer	Sigma-Aldrich, St. Louis (USA)
RNase A	Qiagen, Hilden (GER)
RNase free water	Qiagen, Hilden (GER)
Roti® histol	CarlRoth, Karlsruhe (GER)
RPMI medium 1640 (1X)	Gibco life technologies, ThermoFisher Scientific, Waltham (USA)
SOC medium	Invitrogen, ThermoFisher Scientific, Waltham (USA)
SeaKem® LE agarose	Seakem LE, Biozym Scientific GmbH, Oldendorf (GER)
Skim milk powder	Sigma-Aldrich, St. Louis (USA)
Sodium chloride	Honeywell, Morris Plains (USA)
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, St. Louis (USA)
Sodium hydroxide	Honeywell, Morris Plains (USA)

Tetraacetylethylenediamine (TEMED)	Bio-Rad Laboratories Inc., Hercules (USA)
TGX Stain-Free™ FastCast™ resolver A	Bio-Rad Laboratories Inc., Hercules (USA)
TGX Stain-Free™ FastCast™ resolver B	Bio-Rad Laboratories Inc., Hercules (USA)
TGX Stain-Free™ FastCast™ stacker A	Bio-Rad Laboratories Inc., Hercules (USA)
TGX Stain-Free™ FastCast™ stacker B	Bio-Rad Laboratories Inc., Hercules (USA)
Triton™ X-100	Sigma-Aldrich, St. Louis (USA)
Trizma® base	Sigma-Aldrich, St. Louis (USA)
Trypsin/EDTA	Pan Biotech, Aidenbach (GER)
Tween® 20	Sigma-Aldrich, St. Louis (USA)
Ultramount	Agilent technologies, Santa Clara (USA)
Waymouth's MB 752/1 medium (1X)	Gibco life technologies, ThermoFisher Scientific, Waltham (USA)
Xylol	VWR chemicals, Radnor (USA)

2.1.2 Solutions

All used solutions are listed in table 4.

Table 4: Solutions and their composition.

Solution	**Composition**	**Amount / Volume**
10 % formalin solution	ddH_2O 37 % formalin solution	730 ml 270 ml
10X TBE	Borate acid ddH_2O EDTA Tris base 30 % HCl	51.53 gr 1000 ml 3.72 gr 121.1 gr To pH = 8
10X Running buffer	ddH_2O	990 ml

	Glycine 20 % SDS Tris base	144.2 gr 10 ml 30.22 gr
10X TBST	ddH_2O Sodium chloride Tris 1M Tween 20	790 ml 80 gr 200 ml 10 ml
1X Citrate buffer	Concentrated (10X) antigen retrieval Citra plus solution ddH_2O	100 ml 900 ml
1X PBS	10X PBS buffer pH 7.4 ddH_2O	100 ml 900 ml
1X Running buffer	ddH_2O 10X running buffer	900 ml 100 ml
1X TBST	10X TBST ddH_2O	100 ml 900 ml
1X Transfer buffer	ddH_2O Glycine Methanol Tris base	800 ml 14.42 gr 200 ml 3.02 gr
1X Tris buffer	ddH_2O HCl 1M Sodium chloride Tris base	922 ml 78.6 ml 170 gr 121 gr
1X TBE	ddH_2O 10X TBE	900 ml 100 ml
70 % ethanol	100 % ethanol ddH_2O	700 ml 300 ml
80 % ethanol	100 % ethanol ddH_2O	800 ml 200 ml
90 % ethanol	100 % ethanol ddH_2O	900 ml 100 ml
96 % ethanol	100 % ethanol	960 ml

	ddH_2O	40 ml
Bicinchoninic acid-copper(II)sulphate mix	Bicinchoninic acid copper(II)sulphate	980 µl 20 µl
BSA blocking reagent	Albumin bovine fraction V, pH = 7.0 1X TBST	5 gr 100 ml
Cell lysis buffer	10X Cell lysis buffer Phosphostop EASYpack Proteaseinhibitor cOmplete Tablets EASYpack	10 ml 1 P 1 P
Complete DMEM medium	DMEM (1X) FBS P-S	500 ml 50 ml 5 ml
Complete LB medium	LB Broth (Miller) Ampicillin (c = 100 mg/ml)	500 ml 500 µl
Complete RPMI medium	RPMI-1640 medium (1X) FBS P-S	500 ml 50 ml 5 ml
Complete Waymouth's medium	Waymouth's MB 752/1 medium (1X) Iron (II) sulphate heptahydrate Hydrocortisone L-ascorbic acid P-S	500 ml 225 µg 1 mg 150 mg 5 ml
DNA ladder mix	6X DNA loader Gene ruler™ 50 bp Plus DNA ladder / Gene ruler™ 1 kb Plus DNA ladder RNase free water	1 µl 1 µl 4 µl
EDTA (0,5 M)	EDTA ddH_2O	9,3 g 50 ml

EGTA (0,5 M)	EGTA ddH_2O	1,9 g 20 ml
Eosin solution	3 % acetic acid 3.75 % eosin ddH_2O	0.75 ml 11.25 ml 289.25 ml
Freezing medium	Complete medium (RPMI) FBS DMSO	8 ml 1 ml 1 ml
HEPES (0,5 M)	HEPES (1 M) ddH_2O	2 ml 2 ml
Hypotonic homogenisation buffer	HEPES (0,5 M) Potassium chloride (3 M) EDTA (0,5 M) EGTA (0,5 M) Protease inhibitor DTT (0,5 M) ddH_2O	2 ml 333 µl 20 µl 20 µl 1 25 µl 97,6 ml
Laemmlibuffer	4 X Laemmli sample buffer 2-mercaptoethanol	900 µl 100µl
Matrigel matrix	Corning® Matrigel® Matrix 1X PBS	1 ml 1 ml
Milk blocking reagent	Skim milk powder 1X TBST	5 g 100 ml
Nuclear extraction buffer	HEPES (0,5 M) Glycerol 89% Sodium chloride (5 M) EDTA (0,5 M) Protease inhibitor DTT (0,5 M) ddH_2O	4 ml 28 ml 8 ml 0,2 ml 1 25 µl 59,8 ml
Potassium chloride(3 M)	Potassium chloride ddH_2O	11,2 g 50 ml
RLT buffer	RLT lysis buffer	1 ml

	2-mercaptoethanol	10 µl
Sodium chloride (5 M)	Sodium chloride ddH_2O	14,6 g 50 ml
20 % TritonX	TritonX 100 ddH_2O	20 ml 80 ml
Tris/Triton buffer	ddH_2O 1X Tris buffer 20 % TritonX	945 ml 50 ml 5 ml

2.1.3 Reagent kits

All used reagent kits are listed in table 5.

Table 5: Reagent Kits and their providers and headquarters.

Reagent Kit	**Provider and headquarter**
DNA, RNA and protein purification • Elution buffer • Equilibration buffer • Lysis buffer • Neutralization buffer • NucleoBond® Xtra maxi columns • Resuspension buffer • Wash buffer	Macherey-Nagel, Düren (GER)
GoScript™ reverse transcription system • GoScript™ 5X reaction buffer • GoScript™ reverse transcriptase • $MgCl_2$, 25 mM • Nuclease free water • Oligo (dT) 15 primer • PCR nucleotide mix, 10 mM • Random primers • Recombinant RNasin® ribonuclease inhibitor	Qiagen, Hilden (GER)

QIAshredder • QIAshredder mini spin column	Qiagen, Hilden (GER)
RNeasy® mini kit: • RLT lysis buffer • RPE wash buffer • RW1 wash buffer • collection tubes (1.5ml) • RNeasy mini spin columns	Qiagen, Hilden (GER)
TGX Stain-free Fast Cast Acrylamide Kit, 10% • Resolver A • Resolver B • Stacker A • Stacker B	Bio-Rad Laboratories Inc., Hercules (USA)

2.1.4 Plasmids

All used plasmids are listed in table 7 and 8.

Table 6: Plasmids with their targets, companies and headquarters.

Plasmid ID	**Target gene**	**Company and headquarter**
NM_021180.2-439s1c1 (name in thesis: shGRHL3-1)	GRHL3	Sigma-Aldrich, St. Louis (USA)
NM_021180.2-236s1c1 (name in thesis: shGRHL3-2)	GRHL3	Sigma-Aldrich, St. Louis (USA)
MISSION® pLKO.1-puro eGFP shRNA Control Plasmid DNA	sheGFP	Sigma-Aldrich, St. Louis (USA)
HGLibA_20225 in lentiCRISPRv2	GRHL3	Provided by Johannes Lustig
HGLibB_20254 in lentiCRISPRv2	GRHL3	Provides by Johannes Lustig

Table 7: Plasmids and companies with headquarters.

Plasmid	**Catalogue number**	**Company and headquarter**
lentiCRISPRv2	52961	Addgene, Cambridge (USA)
pLX304	25890	Addgene, Cambridge (USA)
psPAX2	12260	Addgene, Cambridge (USA)
pMD2.G	12259	Addgene, Cambridge (USA)
GRHL3 in pLX304		Kindly provided by Dr. Felix Wezel

2.1.5 CRISPR/Cas9 Oligos

All used CRISPR/Cas9 Oligos are listed in table 9.

Table 9: CRISPR/Cas9 Oligos, their sequence in 5´3´direction and their length in bp.

Target	**Direction**	**Sequence 5' - 3'**
HGLibA_20225	Oligo 1	CACCGATATTGTCCTCCAGCACTGG
	Oligo 2	AAACCCAGTGCTGGAGGACAATATC
HGLibB_20254	Oligo 1	CACCGCAGTAGAGCAGCTGCGCTTC
	Oligo 2	AAACGAAGCGCAGCTGCTCTACTGC

2.1.6 Primers

All used primers had a concentration of 100 pMol and are listed in table 10.

Table 10: Targets of the primers and companies with headquarters.

Target	**Provider and headquarter**
GAPDH	Biomers, Ulm (GER)
GRHL3	Biomers, Ulm (GER)
PTEN	Biomers, Ulm (GER)

UPK1a	Invitrogen, ThermoFisher Scientific, Carlsbad (USA)
UPK1b	Invitrogen, ThermoFisher Scientific, Carlsbad (USA)
UPK2	Eurofins Scientific, Luxemburg
UPK3a	Invitrogen, ThermoFisher Scientific, Carlsbad (USA)
UPK3b	Invitrogen, ThermoFisher Scientific, Carlsbad (USA)

The direction and the sequence in 5' 3' direction of the primers are listed in table 11.

Table 81: Targets of primers and sequence in 5´3´direction.

Target	**Direction**	**Sequence 5' - 3'**
GAPDH	Forward	AGATCCCTCCAAAATCAAGTGG
	Reverse	AAAAGGGTCATCATCTCTGCC
GRHL3	Forward	ATTGACGTGGCTGACTGCAA
	Reverse	GCTCAGACAGTTTACGCCGA
PTEN	Forward	GGCACCGCATATTAAAACGTA
	Reverse	ATGCCATTTTTCCATTTCCA
UPK1a	Forward	GTGGTGGGCCTGCTAGTTG
	Reverse	TATACACGCTACTGGTCGGCT
UPK1b	Forward	CCAAAGACAACTCAACTGTTCGT
	Reverse	AATGCCGCAACAACCAATAATC
UPK2	Forward	ACCAGGTGACAAACCTCGTG
	Reverse	TGTTCCTTCGAGGGAGTGTG

UPK3a	Forward	CGTGGACATGGGGAGTTCTG
	Reverse	TCACGGACGTGTAGGAAGACT
UPK3b	Forward	CCTCTACCATGCGCTTCTCC
	Reverse	ATGTGGTGGGTCATGTAGCG

2.1.7 Antibodies

All used primary antibodies are listed in table 12 and all used secondary antibodies are listed in table 13.

Table 9: Primary antibodies, their antigens, the experiments they were used, the dilutions and companies with headquarters.

Antigen	**Species**	**Clone**	**Experiment**	**Dilution**	**Company and headquarter**
Akt1 (D9R8K)	rabbit	#75692	WB	1:2,000	Cell Signaling Technology, Danvers (USA)
β-Actin	mouse	AC-15	WB	1:10,000	Sigma-Aldrich, St. Louis (USA)
GRHL3	mouse	aa529-578 LS-C31710	WB	1:500	LifeSpan BioSciences, Seattle (USA)
GRHL3	mouse	C-12, sc-398838, IgG2b	WB	1:1,000	Santa Cruz, Dallas (USA)
GRHL3	rabbit	HPA059960	WB	1:250	Sigma-Aldrich, St. Louis (USA)
Multi-cytokeratin	mouse	NCL-L-AE1/AE3	IHC		Leica Biosystems, Wetzlar (GER)

Phospho-Akt (Ser473)	rabbit	#4060 (D9E)	WB	1:2,000	Cell Signaling Technology, Danvers (USA)
Phospho-Akt (Thr308)	rabbit	#13038 (D25E6)	WB	1:2,000	Cell Signaling Technology, Danvers (USA)
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	rabbit	#4370 (D13.14.4E)	WB	1:2,000	Cell Signaling Technology, Danvers (USA)
PTEN	rabbit	#9559 (138G6)	WB	1:1,000	Cell Signaling Technology, Danvers (USA)
Sp1	goat	PLA0307-100UL	WB	1:1,000	Sigma-Aldrich, St. Louis (USA)
TBP	goat	#11401P1	WB	1:1,000	Sigma-Aldrich, St. Louis (USA)

Table 10: Secondary antibodies, the experiments they were used for, the dilution and the companies with headquarters.

Species	**Antigen**	**Conjugate**	**Experiment**	**Dilution**	**Company and headquarter**
chicken	rabbit IgG	HRP	WB	1:2,000	Santa Cruz, Dallas (USA)
goat	Rabbit Ig, Simple stain MAX PO (M)	immunoperoxidase	IHC		Nichirei Biosciences, Tokyo (J)

goat	Mouse Ig, Simple stain MAX PO (R)	immunoperoxidase	IHC		Nichirei Biosciences, Tokyo (J)
horse	mouse IgG	HRP	WB	1:2,000	Cell Signaling Technology, Danvers (USA)
rabbit	goat IgG	HRP	WB	1:2,000	Invitrogen, Rockford (USA)

2.1.8 Cell lines and bacteria

All used cell lines are listed in table 14 and the bacteria are listed in table 15.

Table 11: Cell lines, their cell types, culture conditions and origin.

Cell line	**Cell type**	**Culture conditions**	**Identification number**
HEK 293T	Human Embryonal kidney cell	37 °C, 5 % CO_2, 95 % humidity, Complete DMEM medium	ATCC® CRL-1573™
HT1197	Human carcinoma of the urinary bladder	37 °C, 5 % CO_2, 95 % humidity, Complete RPMI medium + 1% Glutamax + 1% non-essential amino acids	ATCC® CRL-1473™
RT4	Human transitional cell papilloma	37 °C, 5 % CO_2, 95 % humidity, Complete RPMI	ATCC® HTB-2™

		medium	
T24	Human transitional cell carcinoma, G3	37 °C, 5 % CO_2, 95 % humidity, Complete RPMI medium	ATCC® HTB-4™
UMUC-3	Human transitional cell carcinoma	37 °C, 5 % CO_2, 95 % humidity, Complete RPMI medium	ATCC® CRL-1749™

Table 12: Bacteria, their name and company with headquarters.

Bacteria	**Name**	**Company and headquarter**
Escherichia coli	One Shot™ Stbl3™ Chemically Competent *E. coli*	ThermoFisher Scientific, Waltham (USA)

2.1.9 Devices

All used devices are listed in table 16.

Table 13: Devices, their commercial name and companies with headquarters.

Device	**Commercial name**	**Company and headquarter**
Casting stand	MiniPROTEAN®	Bio-Rad Laboratories Inc. Hercules (USA)
Centrifuge	Centrifuge 5417R	Eppendorf, Hamburg (GER)
Centrifuge	Centrifuge 5418R	Eppendorf, Hamburg (GER)
Centrifuge	Centrifuge 5424	Eppendorf, Hamburg (GER)

Centrifuge	Megafuge 1.0 R	Heraeus instruments, Hanau (GER)
Centrifuge	Megafuge 40R	ThermoFisher Scientific, Waltham (USA)
Cycler	Labcycler	Sensquest, Göttingen (GER)
Power supply for gel electrophoresis	EV2310	Biobudget Technologies, Krefeld (GER)
Fully automated rotary microtome	Leica RM2255	Leica, Wetzlar (GER)
Heated paraffin embedding module	Leica EG1150 H	Leica, Wetzlar (GER)
Heatstir	IKAmag RCT	Janke und Kunkel, Staufen (GER)
Horizontal shaker	IKA VIBRAX-VXR	Janke und Kunkel, Staufen (GER)
Horizontal shaker	Rocking Platform	VWR, Radnor (USA)
Hotplate 1092	Hotplate 1092	Severin, Sundern (GER)
Incubator	B5042	Heraeus instruments, Hanau (GER)
Incubator	BBD 6220	Heraeus instruments, Hanau (GER)
Incubator	Certomat® HK	Braun Biotech International, Sartorius AG, Göttingen (GER)
Micro scales	AB184-S-A	Mettler Toledo, Greifensee (CHE)
Microplate spectral photometer	Epoch	Biotek Instruments, Winooski (USA)
Microscope	Axio Imager M2	Zeiss, Oberkochen (GER)
Microscope cell culture	Axiostar Vert A1	Zeiss, Oberkochen

		(GER)
Microscope camera	AxioCam Cm1	Zeiss, Oberkochen (GER)
Microscope camera slides	Axiocam 105 color	Zeiss, Oberkochen (GER)
Microtome	RM2035	Leica, Wetzlar (GER)
Minicentrifuge	Ministar silverline	VWR, Radnor (USA)
Pipettes	Pipetman® G MICRO VOLUME KIT	Gilson, Madison (USA)
Power supply for western blot	Powerpac HV™	Bio-Rad Laboratories Inc. Hercules (USA)
Pressure cooking pot	Cromargan, Stainless steel 18/10	WMF Group, Geislingen/Steige (GER)
q-RT-PCR cycler	Viia 7	Applied biosystems, Foster City (USA)
Scales	EMB 500-1	Kern, Balingen-Frommern (GER)
Sterile bench	MSC ADVANTAGE	ThermoFisher Scientific, Waltham (USA)
Tetra Cell System	MiniPROTEAN®	Bio-Rad Laboratories Inc. Hercules (USA)
Thermomixer	Thermomixer comfort	Eppendorf, Hamburg (GER)
Thermomixer	HTM H200042	HTA Biotech, Bovenden (GER)
Tissue flotation bath	TFB 45	MEDITE, Burgdorf (GER)
Transfer chamber	Transfer chamber	Bio-Rad Laboratories Inc. Hercules (USA)
Ultra-low temperature freezer	Igloo U 830	Telstar, Terassa (ESP)
Vortex	Reax 2	Heidolph Instruments, Schwabach (GER)
Vortex	Vortex Genie 2™	Bender&Hobein AG,

		Zürich (CHE)
Vortex	RS-VA 10	Phoenix Instruments, Napreville (USA)
Vortex	Heidolph Reax 2000	Heidolph Instruments, Schwabach (GER)
RTCA Analyzer	xCELLigence RTCA DP	Agilent technologies, Santa Clara (USA)
Fluorescence and chemiluminescence imaging system	FusionFx	Vilbert Lourmat, Collégien (F)
Freezing container	NALGENE™ Cryo 1 C	ThermoFisher Scientific, Waltham (USA)

2.1.10 Software

All used software are listed in table 17.

Table 14: Software and their companies with headquarters.

Software	**Company and headquarter**
FusionCapt Advance Solo 7 17.01	Vilbert Lourmat, Collégien (F)
GraphPad Prism 6	GraphPad Software, Inc, La Jolla (USA)
ImageJ 1.51r	Public domain
Microsoft Office	Microsoft, Redmond (USA)
xCELLigence RTCA DP	Agilent technologies, Santa Clara (USA)
ZEN 2.3 Pro	Carl Zeiss Microscopy GmbH, Jena (GER)

2.1.11 Consumables

All used consumables are listed in table 18.

Table 15: Consumables and their companies with headquarters.

Consumables	**Company and headquarter**
Biosphere Filter Tips 100 – 1000 µl	Sarstedt, Nümbrecht (GER)
Biosphere Filter tips 2 – 100 µl	Sarstedt, Nümbrecht (GER)
Biosphere Filter tips 2 - 20 µl	Sarstedt, Nümbrecht (GER)
Biosphere Filter Tips 2 - 200 µl	Sarstedt, Nümbrecht (GER)
Cell scrapper 25 cm	Sarstedt, Nümbrecht (GER)
Cell strainer, 70 µm Nylon, White frame	VWR International, Radnor (USA)
CIM-plate 16	ACEA Biosciences, San Diego (USA)
Cover slip	ThermoFisher Scientific, Waltham (USA)
CryoPure Tubes 1.8 ml red	Sarstedt, Nümbrecht (GER)
Culture-Inserts 4 Well	Ibidi, Gräfelfing (GER)
Delicate Task Wipes – White	Kimtech, Dallas (USA)
Disposal bags, PP	MerckMillipor, Darmstadt (GER)
Erlenmeyer flask 1000 ml Borosilicate 3.3	Schott AG, Mainz (GER)
Erlenmeyer flask 500 ml Borosilicate 3.3	Schott AG, Mainz (GER)
Filter 0,2 µm	CarlRoth, Karlsruhe (GER)
Filter 0,45 µm	CarlRoth, Karlsruhe (GER)
Filter tip PP natural 0.1 – 10 µl	Nerbe plus, Winsen/Luhe (GER)
Funnel 100 mm	VWR International, Radnor (USA)
Funnel 125 mm	VWR International, Radnor (USA)
Glass bottle 100 ml Borosilicate 3.3	VWR International, Radnor (USA)
Glass bottle 1000 ml Borosilicate 3.3	VWR International, Radnor (USA)
Glass bottle 2000 ml Borosilicate 3.3	VWR International, Radnor (USA)
Glass bottle 250 ml Borosilicate 3.3	VWR International, Radnor (USA)
Glass bottle 500 ml Borosilicate 3.3	VWR International, Radnor (USA)
Glass slide holder	CarlRoth, Karlsruhe (GER)
Green tissue embedding cassettes	CarlRoth, Karlsruhe (GER)
Haemocytometer, Depth 0,1 mm,	Superior Marienfeld, Lauda-

0,0025 mm², Neubauer	Königshofen (GER)
Inoculator rigide	VWR International, Radnor (USA)
LB agar plates with ampicillin 100	Teknova, Hollister (USA)
Messbecher 100 : 1 ml Boro 3.3	Brand Eterna Duran
Messbecher 2000 : 50 ml Boro 3.3	Brand Eterna Duran
Messbecher 250 : 2 ml Boro 3.3	Brand Eterna Duran
Micro tube 1.5 ml SafeSeal	Sarstedt, Nümbrecht (GER)
Micro tube 2.0 ml SafeSeal	Sarstedt, Nümbrecht (GER)
Mini Trans-Blot Accessories, blotting pads, filter paper	Bio-Rad Laboratories Inc., Hercules (USA)
Mini Trans-Blot Cell Module	Bio-Rad Laboratories Inc., Hercules (USA)
Mini Trans-Blot Foam pads	Bio-Rad Laboratories Inc., Hercules (USA)
Mini-Protean Comb, 10 well	Bio-Rad Laboratories Inc., Hercules (USA)
Mini-Protean Gel releasers	Bio-Rad Laboratories Inc., Hercules (USA)
Mini-Protean Short plates	Bio-Rad Laboratories Inc., Hercules (USA)
Peha-soft nitrile, white, L 8-9	Paul Hartmann AG, Heidenheim (GER)
Plastic slide holder	CarlRoth, Karlsruhe (GER)
Protran Nitrocellulose Transfer Membrane	Whatman, Dassel (GER)
SafeSeal-Tips professional 100 µl	Biozym, Hessisch Oldendorf (GER)
Serological pipette 10 ml	Sarstedt, Nümbrecht (GER)
Serological pipette 2 ml	Sarstedt, Nümbrecht (GER)
Serological pipette 5 ml	Sarstedt, Nümbrecht (GER)
Serological pipette 50 ml	Sarstedt, Nümbrecht (GER)
Single-use syringes, 2-piece, 10 ml	B. Braun, Melsungen (GER)
Superfrost Plus™ Microscope slides	ThermoFisher Scientific, Waltham (USA)

TC Dish 100, Standard	Sarstedt, Nümbrecht (GER)
TC Dish 150, Standard	Sarstedt, Nümbrecht (GER)
TC Flask T25, Stand., Vent. Cap	Sarstedt, Nümbrecht (GER)
TC Flask T75, Stand., Vent. Cap.	Sarstedt, Nümbrecht (GER)
TC Plate 96 Well, Standard, F	Sarstedt, Nümbrecht (GER)
TC-Platte 6 Well, Standard, F	Sarstedt, Nümbrecht (GER)
Tube 15 ml, 120x17 mm, PP	Sarstedt, Nümbrecht (GER)
Tube 50 ml, 115x28 mm, PP	Sarstedt, Nümbrecht (GER)

2.2 Methods

2.2.1 Cell culture

The cells were cultured in 75 cm² tissue culture flasks (T75). T24, UMUC3 and RT4 were cultured in RPMI with 10% fetal calf serum (FCS) and 1% Penicillin/Streptomycine. HT1197 was cultured in RPMI with 10% fetal calf serum (FCS), 1% Penicillin/Streptomycine, 1% Glutamax and 1% non-essential amino acids. They were kept in incubators with 5% CO_2 and 37 °C. Cell growth was monitored with a light microscope. Based on experimental needs and confluence the cells were split in new flasks. Dulbecco's Phosphate Buffered Saline (PBS) and Trypsin were prewarmed to 37 °C. The medium was removed and the cells were washed with 5 ml PBS. 2 ml Trypsin were added and the cells were incubated at 37 °C for 5 minutes. 8 ml medium were added and the cells were distributed to a new flask according to the needs. The medium in the new flask was filled up to 10 ml.

2.2.2 Transformation

One Shot Stbl3 chemically competent *E. coli* were thawed on ice and 1000 ng plasmid were added. The vial was incubated 30 minutes on ice and heat shocked for 45 seconds at 42 °C. It was incubated for 2 minutes on ice. 250 µl of pre-

warmed S.O.C. medium were poured to the vial. The vial was incubated for 1 hour at 37 °C shaking at 230 rounds per minute (rpm). 50 µl and 100 µl were spread on two pre-warmed Ampicillin selective plates. The remaining transformation mix was stored at 4 °C and the plates were incubated at 37 °C over night.

2.2.3 Maxi Prep

One colony from the transformation was added to 300 ml LB Broth medium with 100 µg Ampicillin per ml and incubated for 16 hours at 37 °C shaking at 220 rpm. The bacterial culture was spread to 6 50 ml falcons and centrifuged 10 minutes at 6000 g at 4 °C and the supernatant was discarded. The pellets were resuspended in 12 ml Resuspension buffer with RNase A and transferred in one 50 ml falcon. 12 ml Lysis buffer were added by pipetting it on the wall. The falcon was inverted 5 times and incubated 5 minutes at room temperature. The NucleoBond Xtra column and the NucleoBond Xtra filter were equilibrated with 25 ml Equilibration buffer. 12 ml Neutralization buffer were added to the suspension by pipetting it on the wall of the falcon. The falcon was inverted 10 times. It was centrifuged for 15 minutes at 5000 g at 4 °C. The supernatant was poured on the filter and flowed completely through the column into a 50 ml falcon. The filter and the column were washed with 15 ml Equilibration buffer. The filter was discarded. The column was washed with 25 ml Wash buffer by pipetting it to the wall. The falcon was replaced with another 50 ml falcon and 15 ml pre-warmed Elution buffer were poured to the column. When the Elution buffer flowed completely through the column, the column was discarded. 10.5 ml pre-cooled isopropanol were poured to the eluted plasmid DNA. It was vortexed and centrifuged for 30 minutes at 15000 g at 4 °C. The supernatant was discarded. The pellet was washed with 5 ml 70% ethanol. It was centrifuged 5 minutes at 15000 g at room temperature. The ethanol was removed with a pipette. The DNA was kept for 5 minutes at room temperature for drying. The DNA was resuspended in 200 µl sterile water by shaking for 10 minutes at 300 rpm at 25 °C.

2.2.4 Nucleotide quantitation

Spectrophotometric analysis was used for RNA and DNA quantitation. It was performed in duplicates. To have a blank 2 µl of RNase free water were added to the microplate spectral photometer and measured. Then 2 µl of the nucleotide were added and measured.

2.2.5 Enzymatic digestion

1 µg plasmid, 5 µl 10X smart buffer and 1 µl of two enzymes each were mixed and filled up to 20 µl with Nuclease free water. It was shaken 1 hour at 200 rpm at 37 °C.

2.2.6 Gel electrophoresis

A 1% agarose gel was prepared by mixing 125 ml 1X TBE with 1.3 g low melting temperature agarose in a Erlenmeyer flask. It was heated 3 minutes in the microwave and cooled down to 55 °C by let water flow on the outer wall of the Erlenmeyer flask. 12 µl peqGreen were added. It was poured in an insert and a comb with 8 1.5 mm wells was inserted. It was kept for drying for 30 minutes. The needed amount of plasmid was diluted to 18 µl and 3 µl loading dye were added. 12 µl 1 KB Gene ruler were used as marker. The samples were separated by running for 3 h at 100 V. Pictures were taken with the Fusion FX.

2.2.7 Transfection and infection

HEK cells were seeded to a 10 cm² plate to get 50% confluency on the next day. On the next day a mixture of 500 µl DMEM pure, 1.8 µg pSPAX2, 300 ng pMD2, 3 µg expression plasmid and 30 µl PEI was prepared. The DMEM complete of the HEK cells was changed and the mixture was poured to the plate. It was incubated for 24 hours at 37 °C with 5% CO2. The transfection reagent was replaced with 10

ml DMEM complete and the plate was incubated for 24 hours at 37 °C with 5% CO2. The target cells were seeded in T75 flasks to get 40-50% confluency in two days. The virus was collected with a syringe and filtered with a 0.45 µm filter into a 50 ml falcon. It was stored at 4 °C. 10 ml DMEM complete were added to the HEK cells. The plate was incubated again for 24 hours at 37 °C with 5% CO2. The virus was again collected with a syringe and filtered with a 0.45 µm filter into the same 50 ml falcon. A mixture of 4 ml target cells' medium, 1 ml virus and 40 µg Polybren was prepared. The medium of the target cells was replaced with the mixture. The cells were incubated again for 24 hours at 37 °C with 5% CO2. The virus was aliquoted in cryo tubes and stored at -80 °C. On the next day the cells were washed 3 times with 5 ml PBS and 10 ml of the target cells medium were added. The cells recovered 2 days before the selection was started with 2 µg/ml puromycin for the cells transduced with shRNA and CRISPR/Cas9 plasmids and 10 µg/ml for cells with GRHL3 overexpression.

2.2.8 Freezing cells

Cells were washed with PBS and detached by trypsin. They were centrifuged 3 minutes at 1200 rpm and the supernatant was discarded. The pellet was resuspended in the freezing medium consisting of 90% FCS and 10% DMSO and 1 ml was filled in one cryo tube. The cryo tubes were put in isopropanol and in the -80°C fridge overnight. At the next day the tubes were frozen in liquid nitrogen.

2.2.9 Thawing cells

The cryo tubes with the frozen cells were thawed in a 37 °C water bath for 10 seconds. 1 ml medium were added to the cells and they were centrifuged for 3 minutes at 1200 rpm at room temperature. The supernatant was discarded, the pellet was resuspended in 10 ml pre-warmed medium and poured into a T75 flask. On the next day the medium was changed without washing the cells.

2.2.10 Counting cells

For counting the cells a hemocytometer was used. 10 µl of the cells were added and the number of cells x was counted using a light microscope. With the formula $y = x \div 4 \times 10000$ the number of cells in one millilitre y was calculated.

2.2.11 Preparation of cell lysates

Cells were washed with PBS an detached either by trypsin or by a cell scrapper. They were centrifuged 3 minutes at 1200 rpm and the supernatant was discarded. The pellet was resuspended in RIPA buffer which had three times the volume of the pellet. The cells were transferred in a 1.5 ml Eppendorf tube and destroyed mechanically. The cells were centrifuged 45 minutes at 14000 rpm at 4 °C. The supernatant was transferred in another 1.5 ml Eppendorf tube and stored at -80 °C.

2.2.12 Preparation of nuclear extracts

The cells were detached by trypsin and centrifuged 5 minutes at 800 rpm at 4 °C. The supernatant was discarded and the pellet resuspended in 0.5 ml PBS. The cells were transferred to a 1.5 ml Eppendorf tube and centrifuged again 5 minutes at 800 rpm at 4 °C. The supernatant was discarded and the pellet was resuspended in hypotonic homogenisation buffer which had two times the volume of the pellet. It was incubated on ice for 15 minutes. One twentieth of the pellet's volume of 10% NP-40 was added. It was vortexed for 10 seconds and centrifuged one minute at 15000 g at 4 °C immediately. The supernatant was discarded and 1.25 times of the pellet's volume of Nuclear extraction buffer were added to the pellet without resuspending. The Eppendorf tube was shaken for 20 minutes at 1200 rpm at 4 °C. It was centrifuged for 8 minutes at 15000 g at 4 °C. The supernatant was transferred to another Eppendorf tube und frozen at -80 °C.

2.2.13 BCA Assay

The BCA assay was done in duplicates or triplicates. The protein was diluted 1:10. 10 µl were added to a 96 well plate. Copper (II) sulphate solution and bicinchoninic acid were mixed 1:50 and 100 µl were added to the diluted protein in the 96 well plate. The plate was incubated for 45 minutes at 37 °C. The absorption spectrum was measured at 592 nm wavelength spectrophotometrically. The concentration was calculated using the BSA calibration curve.

2.2.14 Western Blot

60 µg of nuclear extracts or 25 µg of cell lysates were diluted to 16 µl and 4 µl Laemmli buffer with 5% 2-mercaptoethanol were added. It was boiled for 10 minutes at 96 °C and centrifuged short. A gel was poured with 3 ml of Resolver A, 3 ml of Resolver B, 30 µl of 10% APS and 3 µl of TEMED with 1 mm glass plates. A mixture of 1 ml of Stacker A, 1 ml of Stacker B, 10 µl of 10 % APS and 2 µl of TEMED were added on top. A 10 well comb was put in the Stacker. 4 µl of a protein molecular mass marker and the samples were loaded. They were separated for 4 hours with 60 V in 1X Running buffer. The protein was transferred to the membrane that was equilibrated for 5 minutes in methanol. The transfer sandwich was arranged beginning at the cathode with Whatman paper, gel, membrane and Whatman paper. The transfer was done on ice for 2 hours at 300 mA with 1X Transfer buffer. The membrane was blocked for 1 hour at room temperature with either 5% milk in 1X TBST, 5% BSA in 1X TBST or Pierce Protein-free T20 blocking buffer depending on the primary antibody used later. The membrane was incubated with the primary antibody overnight at 4 °C. On the next day it was washed 3 times in 1X TBST for 10 minutes and incubated for 2 hours with the secondary antibody at room temperature. It was washed 3 times in 1X TBST for 10 minutes. For detection 300 µl of Ace glow chemiluminescence substrate were used. Detection was performed with the Fusion Fx. The Western Blots were analysed by measuring the densities of the bands and comparing them to the parental cell lines. These relative densities were divided by the relative densities of the β-actin of each cell line to get adjusted densities.

2.2.15 RNA isolation

The cells were washed with PBS. 700 µl RLT buffer with 1% 2-mercaptoethanol was added and transferred to a 1.5 ml Eppendorf tube. It was vortexed and centrifuged short at 1500 rpm. The centrifuge was 20 °C in all centrifugation steps. The suspension was poured to a QIA shredder. It was centrifuged at 14000 rpm for 2 minutes. The column was discarded. The passage was mixed with 350 µl 70% ethanol and poured to a new column. It was centrifuged at 10000 g for 15 seconds. The passage was discarded and 700 µl RW1 buffer were poured to the column. It was centrifuged at 10000 g for 15 seconds. The passage was discarded and 500 µl RPE buffer were poured to the column. It was centrifuged at 10000 g for 15 seconds again and 500 µl RPE buffer were poured to the column. The column was centrifuged at 10000 g for 2 minutes. The columns were transferred into new collection tubes immediately. They were centrifuged full speed for 1 minute. The columns were transferred into a 1.5 ml Eppendorf tube. 20 µl of RNase free water were added directly on the membrane of the column. It was centrifuged at 8000 g for 1 minute. The columns were discarded and the Eppendorf tubes were frozen at -80 °C.

2.2.16 Reverse transcription

The reverse transcription was done on ice. 100 ng of RNA were poured in an Eppendorf tube. Since the RNA should be in more than 3 µl and not less than 1 µl, dilutions were prepared. The 100 ng of RNA were filled up to 3 µl with RNase free water. It was done with one negative control every time the technique was performed. In the negative control the RNA was replaced by RNase free water. 1 µl of OligodT Primer and 1 µl of Random Primer were added. It was incubated 5 minutes at 70 °C. 15 µl master mix consisting of 6.1 µl Nuklease free water, 4 µl Go Script 5X Reaction buffer, 2.4 µl $MgCl_2$, 1 µl PCR Nucleotide mix, 1 µl Go Script Reverse Transkriptase and 0.5 µl Recombinant RNasin Ribonuclease Inhibitor were poured in the Eppendorf tube. It was annealed 5 minutes at 25 °C, extended 1 hour at 42°C and inactivated 15 minutes at 70 °C. The cDNA was frozen at -80 °C.

2.2.17 Q-RT-PCR

The q-RT-PCR was done on ice. The negative control of the Reverse transcription was used and it was done with one negative control where the cDNA was replaced by Nuclease free water. For every cell line a GAPDH primer and a primer for the gene of interest were used. Both were done in triplicates. The forward and reverse primers were mixed 1:10. 8 µl mastermix consisting of 5 µl iTaq Universal SYBR Supermix, 2 µl RNase free water and 1 µl forward/reverse primer mix were poured in the wells of a 96 well plate. The cDNA was diluted 1:10 and 2 µl were added to the mastermix in the 96 well plate. The plate was sealed and the qPCR was done with the ViiA7 with following conditions: the hold stage was done with 2 minutes at 50 °C and 10 minutes at 95 °C. The PCR stage was done with 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. The melting curve was done with 15 seconds at 95 °C, 1 minute at 60 °C, 30 seconds at 95 °C and 15 seconds at 60 °C. Then it was cooled down to 4 °C.

2.2.18 Wound healing assay

The cells were seeded to get 80-90% confluency on the day of the beginning of the assay. A IBIDI 4 well culture insert was placed in a 6 well plate. 60000 cells of RT4 and 50000 cells of all the other cell lines were seeded in each quarter. The cells were counted using a haemocytometer. The cells were cultured overnight. The inserts were removed with a sterile tweezer on the next day. The cells were washed with 2 ml PBS and 2 ml new medium were added. Pictures of 4 different spots in each well were taken on 10 times magnification after 0 hours, 3 hours, 6 hours, 9 hours, 12 hours, 24 hours, 48 hours, 72 hours and 96 hours or until the wounds were closed. The pictures were analysed with imagej and the plugin MRI_Wound_Healing_Tool.ijm. The plugin measured the area of the wounds in pixels automatically. Those measurements were used to calculate the percentage wound’s size compared with the wound’s size at 0 hours.

2.2.19 Continuous Monitoring of Cell Impedance – Real time cell analyzer (RTCA)

Two days prior to the start the cells were seeded to have 80% confluency in T75 flasks. One day prior to the start the cells standard culture medium was replaced by RPMI pure to starve the cells. The experiment was always done with four replicates using the CIM-Plate 16. The electrodes of the upper chamber were coated with 30 µl of 1% gelatine in 1X PBS and incubated for 40 minutes at room temperature. They were washed with sterile water gently without touching the electrodes. The pre-cooled Matrigel was diluted 1:40 in pre-cooled RPMI pure. The Matrigel was kept on ice the whole time. 50 µl of the Matrigel solution were added to the centre of the first 4 wells of the upper chamber. 30 µl were removed immediately. That was repeated for the remaining wells. It was incubated 4 hours at 37 °C. 160 µl of target cells' medium were added to each well of the lower chamber and 40 µl of RPMI pure were added to each well of the upper chamber. The CIM-plate 16 was placed in the RTCA DP Analyzer for 1 hour at 37 °C. Then a background measurement was performed by starting step 1 (1 minute and 1 sweep) in RTCA software. The cells were washed with 5 ml PBS and incubated with 2 ml pre-warmed Trypsin for 5 minutes at 37 °C. 8 ml medium were added and the cells were counted using a haemocytometer. They were centrifuged 3 minutes at 1200 rpm at room temperature. The supernatant was discarded and the pellet was resuspended with medium to have 400000 cells per ml. 100 µl were added to each well of the upper chamber. Bubble entrapment was avoided. To 4 wells RPMI pure was added as a negative control. The CIM-plate 16 was left in the culture hood for 30 minutes. It was placed in the RTCA DP Analyzer in the incubator. Step 2 in the RTCA software was started with 10 minute intervals for 144 sweeps and then 15 minute intervals for 100 sweeps. After stopping the measurement the Cell Index curves were analysed. The Cell Index was calculated using this formula: $Cell\ index = (impedance\ at\ time\ point\ n - impedance\ in\ the\ absence\ of\ cells) \div nominal\ impedance\ value$

2.2.20 Porcine bladder organoid model

The porcine bladder was kindly supplied by a local abattoir (Ulmer Fleisch GmbH). Connective tissue, urethra and perivesical adipose tissue were removed with a scissor. The bladder was opened and washed three times with PBS containing 3% P/S. It was cut in three pieces and incubated with 0.5% Dispase II overnight at 4 °C. The urothelium was shed by tweezer, washed with PBS containing 3% P/S and downsized to 1 cm^2. Pieces with shedding and without shedding were placed in tissue embedding cassettes and incubated in 10% formalin solution as controls. Those pieces were placed in 70 µm cell strainer inserts with the inner surface of the bladder up. The inserts were placed in 6 well plates. Each well was filled with 3 ml complete Waymouth's medium. The tissue pieces were kept in culture overnight to exclude contamination. Contaminated pieces were discarded immediately. The cells were counted using a haemocytometer. One million cells were centrifuged for 3 minutes at 1200 rpm. The pellet was resuspended in 100 µl RPMI and seeded on the tissue pieces. Everything was kept in the incubator for 10 days at 37 °C with 5% CO_2. The medium was changed every 2 days. Afterwards the tissue pieces were placed in tissue embedding cassettes and incubated in 10% formalin solution for 4 days. The embedding was started with incubating with 70% ethanol for 1 hour at 25 °C. It was incubated three times with 96% ethanol for 1 hour at 25 °C and three times with 100% ethanol for 1 hour at 25 °C. It was cleared with xylene three times for 1 hour at 25 °C and incubated two times for 1 hour at 56 °C. On the next day the tissue pieces were cut in the middle and placed in paraffin so cutting could begin where the pieces were cut. 3 µm thick slices were cut with a microtome. The slices were put in a 37 °C water bath. 3 slices were placed on one microscope slide and dried overnight at 80 °C.

2.2.21 HE staining

The microscope slides with the porcine bladder tissue were incubated for 15 minutes and again 10 minutes with Roti histol. They were washed twice with 100% ethanol for 2 minutes and again with 90 % ethanol, 80 % ethanol and 70% ethanol twice for two minutes each. The slides were washed with distilled water for two

minutes twice. Incubation with haemalm took 7 minutes. They were washed with running tap water for 10 minutes and with HCl water for 3 seconds. The slides were washed 10 minutes in tap water. They were incubated with eosin with 3% acetic acid for 4 minutes and dipped 3 times in distilled water fast. Washing twice for 1 minute in 90% ethanol and three times for 30 seconds in 100% ethanol followed. The slides were dipped 10 times in Roti histol twice. Entellan was used to adhere the cover slips to the slides. They were kept under the hood for drying overnight.

2.2.22 Immunohistochemistry

The slides were placed in a glass slide holder. The slides were deparaffinized with Roti histol twice for 10 minutes, followed by washing 3 times with 100% ethanol for 5 minutes. The slide holder was placed in 70% ethanol for 5 minutes and washed twice for 5 minutes in distilled water. The slides were placed in a plastic slide holder and immersed in 1X citrate buffer in a ceramic insert. The lid was closed and everything was placed in a pressure cooking pot with 1.5 cm distilled water in it. The pot was heated with a hot plate. When steam escaped the power of the hot plate was reduced and everything was boiled for 20 minutes. The lid was cooled with running tap water. The lid of the pot and the ceramic insert were opened and everything was kept for cooling for 20 minutes. The slides were placed in the glass slide holder and shaken for 10 minutes in distilled water, followed by shaking in Tris/Triton buffer for 10 minutes. The corners of cover slips were dipped in liquid paraffin so there was a gap between the cover slips and the slides. These cover slips were placed on the slides. They were incubated with peroxidase blocking solution for 20 minutes in a chamber with high humidity, followed by shaking twice with Tris/Triton buffer for 10 minutes. The slides were dried with Kleenex and again covered with the cover slips. They were incubated with the primary antibody in a chamber with high humidity overnight at 4 °C. On the next day they were shaken twice with Tris/Triton buffer for 10 minutes and incubated with the secondary antibody for 10 minutes in a chamber with high humidity. Washing twice with Tris/Triton buffer for 10 minutes followed. The slides were incubated for 30 minutes with a few drops of AEC in the chamber with high humidity. After 15

minutes AEC was poured on the slides again. They were washed for 10 minutes with distilled water twice and stained with haemalm for 10 seconds. The slides were dipped in distilled water twice and washed with running tap water followed by dipping them again in distilled water twice. One drop of Ultramound was added to each tissue piece and everything was kept for drying overnight. On the next day cover slips were adhered to the slides with Entellan.

2.2.23 Statistical analysis

GraphPad Prism 6 was used for calculating the statistics and graphic creation. Experiments with two groups were analysed with a student's t test. Experiments with three or more groups were analysed with a one-way Anova test. Migration assay was analysed with multiple student's t tests. The significance level was 5 %.

3. Results

3.1 GRHL3 expression in human bladder cancer cell lines

We intended to investigate the role of *GRHL3* in bladder cancer. Therefore, we examined the mRNA expression in four different cell lines. RT4 is a human urothelial papilloma cell line, T24 (G3), UMUC3 and HT1197 are urothelial carcinomas.

To investigate the mRNA level q-RT-PCRs were performed in triplicates at five different time points. The results show readily detectable levels of *GRHL3* mRNA in the non-invasive cell line RT4, but complete absence in the highly invasive bladder cancer cell lines T24 and UMUC3 (Figure 1). Interestingly, a relatively moderate GRHL3 expression was observed in the invasive HT1197 cell line. Although it cannot be excluded that the differences in *GRHL3* mRNA levels are owing to the histo-pathological features of the tumour origin, the above q-RT-PCR results indicate that *GRHL3* expression correlates inverse with the invasive capacities of the cell lines. This was taken as a starting point for the overexpression respectively knock down of *GRHL3* and further experiments.

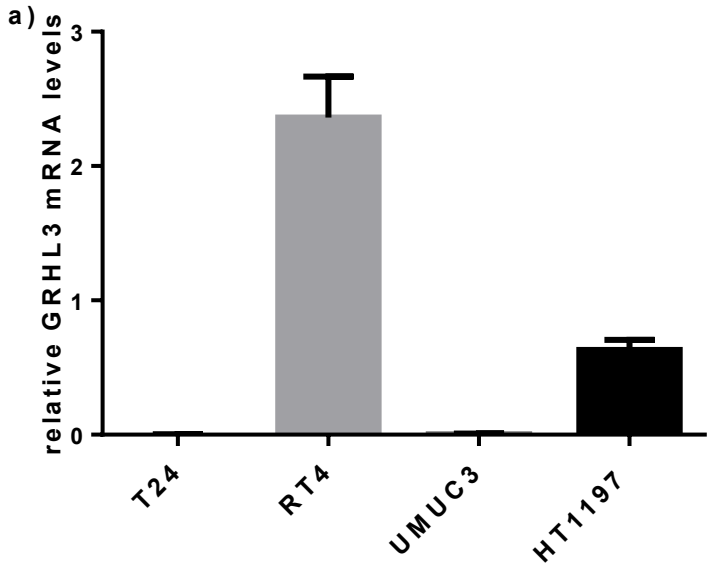


Figure 1: Grainyhead-like 3 transcription factor (GRHL3) messenger RNA (mRNA) expression of the parental cells in five experiments with triplicates. a) T24 and UMUC3 have no expression of GRHL3 on mRNA level. RT4 has the highest expression. HT1197 has a moderate expression. P-values: RT4 vs. T24 <0,0001, UMUC3 vs. T24 0,9233, HT1197 vs. T24 <0,0001, UMUC3 vs. RT4 <0,0001, HT1197 vs. RT4 <0,0001, HT1197 vs. UMUC3 <0,0001.

3.2 Overexpression and knock-down of GRHL3 in different cell lines

Based on this observation, the impact of *GRHL3* on the invasiveness, migration potential, differentiation and signal transduction proteins of bladder cancer cell lines was further studied. Therefore, *GRHL3* was ectopically expressed in the T24 und UMUC3 cell lines that lacked endogenous *GRHL3*. Conversely, *GRHL3* expression was knocked down in RT4 cells. Because the expression in HT1197 was moderate, we decided to express it ectopically and knock it down in this cell line, so we could see if only the overexpression or the knock down or both influence HT1197. This would give a hint of the role of GRHL3 in bladder cancer. As control, we transduced the cells with empty plasmids. pLX304 was used for the overexpression, pLKO.1-puro eGFP shRNA was used for the knock-down.

To determine the expression at mRNA level in T24 after the ectopic expression, a *GRHL3*-specific primer was used in q-RT-PCR in triplicates in three different experiments. *GAPDH* was used as housekeeping gene. In q-RT-PCR a significant overexpression was shown (Figure 2a). These data were substantiated by Western Blot and a overexpression of GRHL3 on protein level was shown (Figure 2b). But GRHL3 was detectable in only one Western Blot overall. In the Western Blot are unspecific bands seen right above the correct GRHL3 band, but the antibody has been verified by my colleague Johannes Lustig. In the following thesis the cell lines are called T24 parental, T24 control and T24 GRHL3.

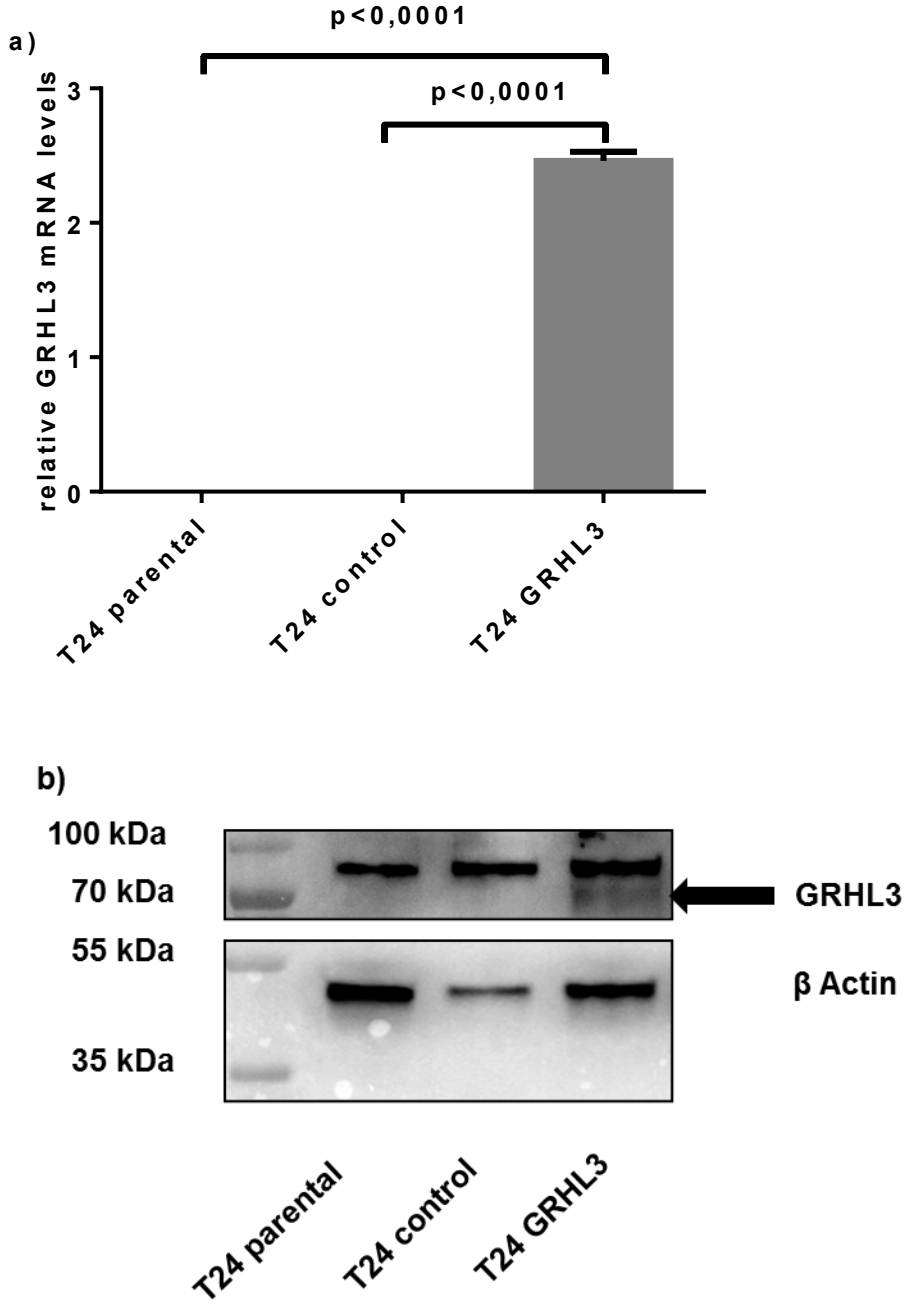


Figure 2: Grainyhead-like 3 protein homolog (GRHL3) protein expression in T24 after overexpression. a) Grainyhead-like 3 transcription factor (GRHL3) expression determined by quantitative real time polymerase chain reaction (q-RT-PCR) relative to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is significant (p<0,0001) higher in the cells with GRHL3 overexpression (N=3). b) The cells with GRHL3 overexpression show a higher protein level in Western Blot. The GRHL3 band is indicated with a black arrow. Above the band are unspecific bands.kDa = kilo Dalton.

In RT4 *GRHL3* was knocked down using CRISPR/Cas9 and shRNA. With shRNA a significant knockdown was achieved with two different plasmids and proven in q-RT-PCR. It was performed in triplicates in four independent experiments. Interestingly, RT4 control had a higher expression than RT4 parental, but not

significant (Figure 3). In Western Blot GRHL3 was not detectable, thus proving the knock-out with shRNA on protein level and the two CRIPSR/Cas9 plasmids was not possible. In the following thesis the cell lines are called RT4 parental, RT4 control, RT4 shGRHL3-1, RT4 shGRHL3-2, RT4 gGRHL3 20225 and RT4 gGRHL3 20254.

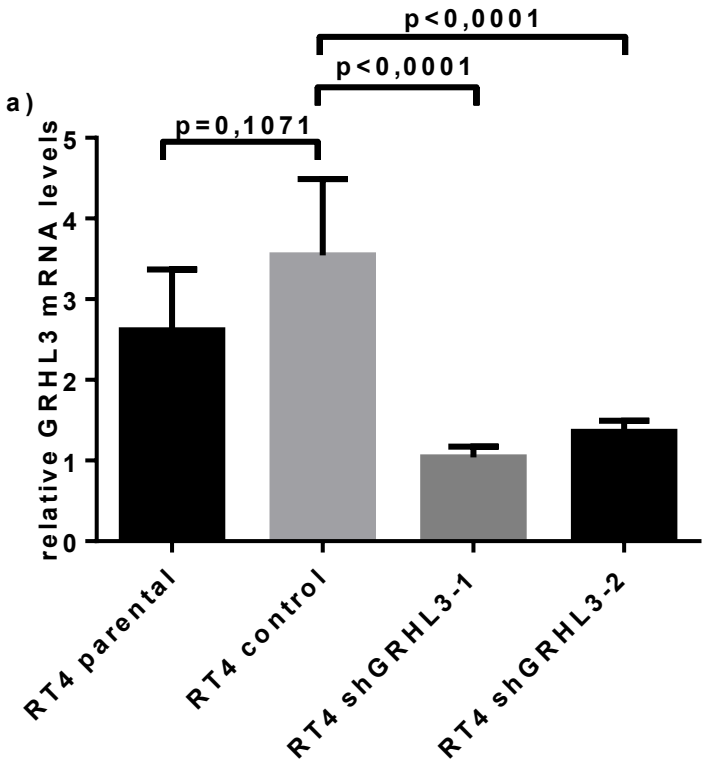


Figure 3 Grainyhead-like 3 transcription factor (GRHL3) messenger RNA (mRNA) expression in RT4 after knockdown with small hairpin RNA (shRNA). a) GRHL3 expression determined by quantitative real time polymerase chain reaction (q-RT-PCR) in RT4 is significant lower after the knockdown with shRNA (N=4).

In UMUC3 and HT1197 a proper overexpression of GRHL3 was not possible (data not presented).

The knockdown with two different shRNA plasmids in HT1197 was successful. Three q-RT-PCRs with triplicates were performed. *GRHL3* was significantly lower expressed in the two cell lines with the shGRHL3 plasmids than in the control (Figure 4). In the following thesis the cell lines will be called HT1197 parental, HT1197 control, HT1197 shGRHL3-1 and HT1197 shGRHL3-2.

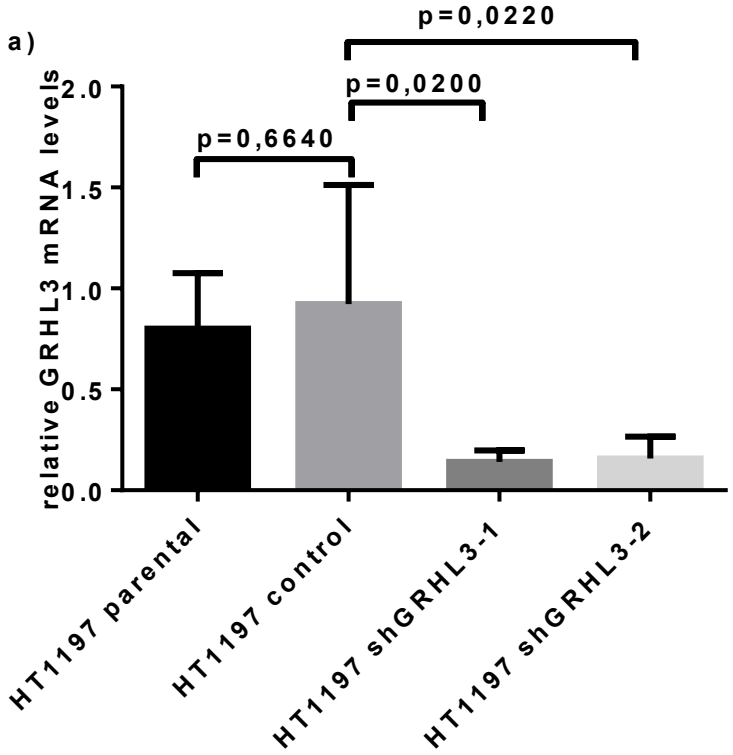


Figure 4: Grainyhead-like 3 transcription factor (GRHL3) messenger RNA (mRNA) expression in HT1197 after knockdown with small hairpin RNA (shRNA). a) GRHL3 expression determined by quantitative real time polymerase chain reaction (q-RT-PCR) in HT1197 is significant lower after the knockdown with shRNA (N=3).

3.3 Invasiveness of bladder cancer cell lines

To investigate the role of GRHL3 in cancer cell invasion, T24 cells with GRHL3 overexpression and RT4 cells with GRHL3 downregulation were investigated in RTCA analysis and the porcine bladder organoid invasion model.

The Continuous Monitoring of Cell Impedance experiment measures the impedance between electrodes, that changes due to cells invading through a membrane and attaching to the electrodes. Then the cell index is calculated: $Cell\ index\ = (impedance\ at\ time\ point\ n - impedance\ in\ the\ absence\ of\ cells) \div nominal\ impedance\ value$.

With RT4 cells the experiment was done once in a quadruplicate with cells with the CRISPR/Cas9 knock out. The RT4 cells showed no increase in the cell index and thus no invasion at all and the cell index curve was close to 0 all the time. This

result was consistent for the cells with the CRISPR/Cas9 knock out. Loss of GRHL3 did not seem to have an influence in invasion (Figure 5).

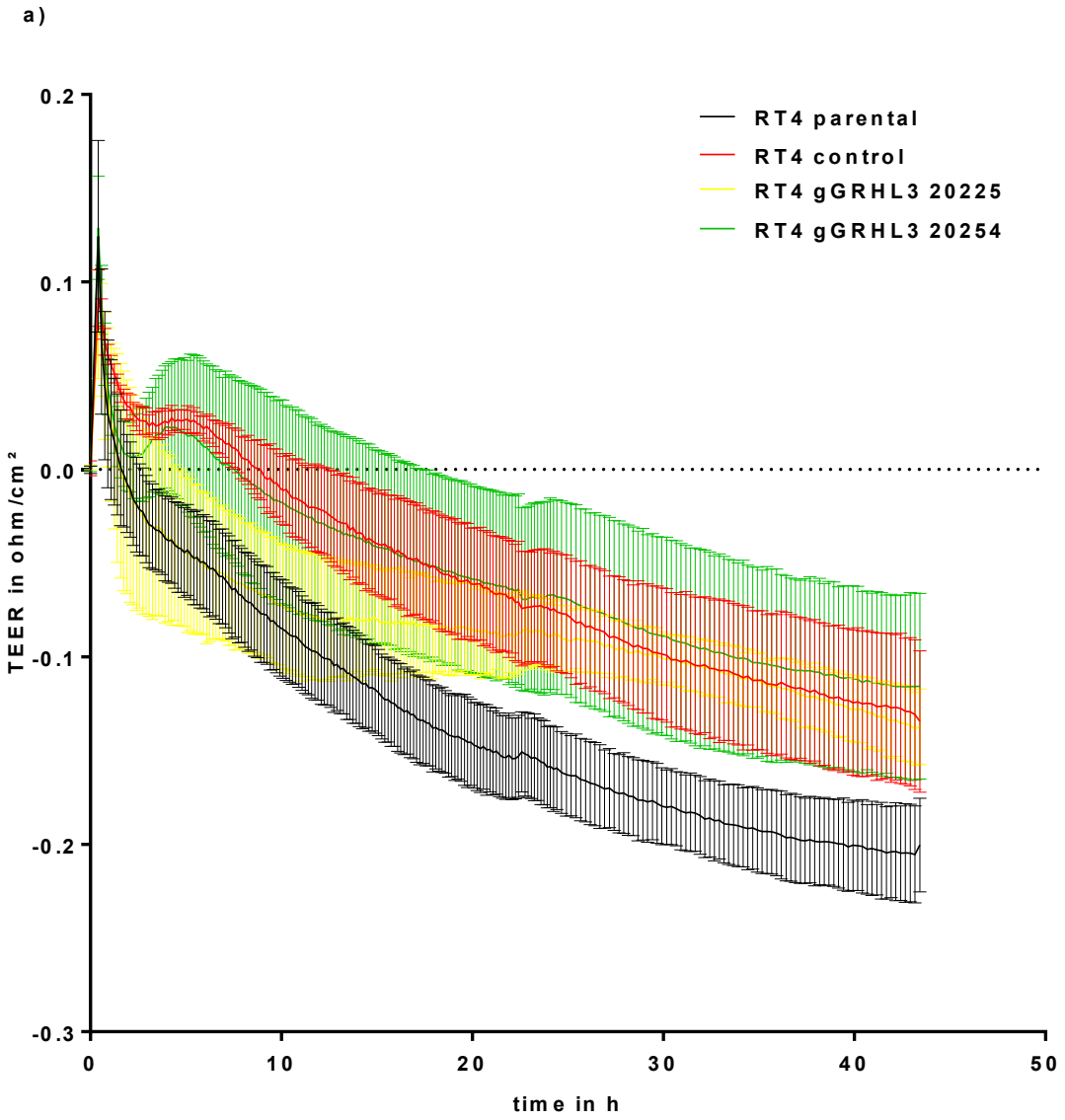


Figure 5 Continuous Monitoring of Cell Impedance of RT4 with Real Time Cell Analyzer (RTCA). TEER = Transepithelial electrical resistance impedance. a) RT4 showed no invasion and the knockout of Grainyhead-like 3 transcription factor (GRHL3) did not alter the invasiveness of the cell line.

The same experiment was repeated with T24 once with a quadruplicate. In contrast to RT4, T24 parental showed an increase of the cell index that reached a plateau after round about 35 hours. The T24 GRHL3 had a significant faster increasing cell index than T24 control and thus a faster invasion especially in the

first 25 hours (Figure 6). Interestingly, this indicates a oncogenic effect of *GRHL3* in bladder cancer on invasive cells that already do not express *GRHL3*.

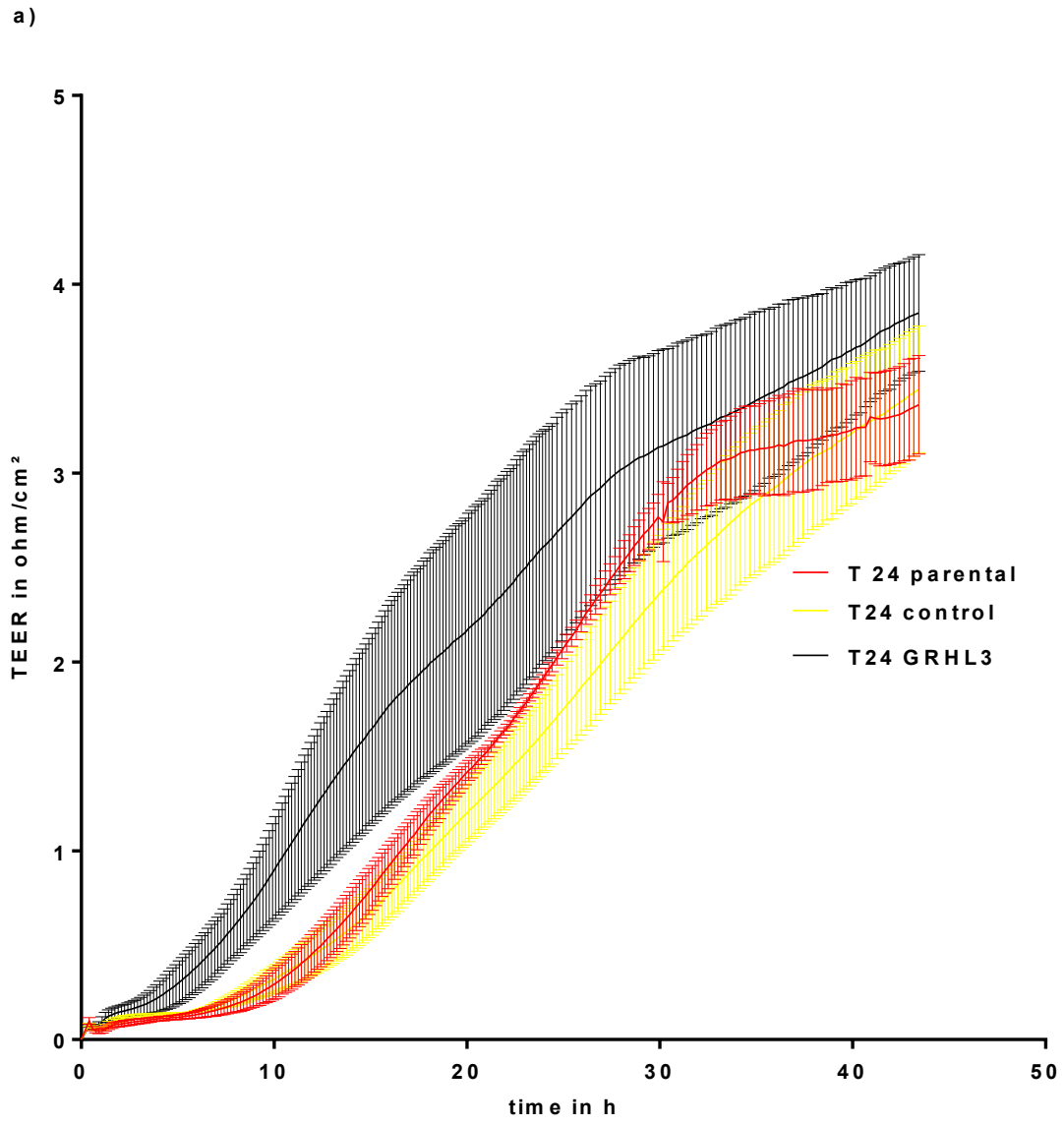


Figure 6 Continuous Monitoring of Cell Impedance of T24 with Real Time Cell Analyzer (RTCA). TEER = Transepithelial electrical resistance impedance. a) T24 shows invasion in this experiment. Especially in the beginning, T24 GRHL3 shows a significant faster invasion than T24 control. P-values: T24 control vs. T24 parental: p=0,2324; T24 control vs. T24 GRHL3: P<0,0001.GRHL3 = Grainyhead-like transcription factor.

To investigate if the cell lines behave the same way in tissue, the porcine bladder organoid model was performed (Figure 7 and Figure 8). We conducted this experiment because it can show invasion in direct contact with tissue over several

days in a manner that is closer to in vivo than the previous experiment. It was conducted in triplicates with T24 and RT4. Porcine bladder was used, the urothelium was scrapped and different cell lines were seeded on it. HE staining and immunohistochemistry were used to compare these bladder samples with normal porcine bladder probes. Unfortunately, RT4 shGRHL3-1 and RT4 shGRHL3-2 were contaminated with fungi, thus only the T24 cells could be analysed. Some technical problems did not permit the immunohistochemistry analysis of the probes, so only the HE staining was analysed. The slides with tissue pieces without scrapping were analysed. Between 30% and 60% of the inner surface of the bladder were covered with urothelium that had between three and ten layers and a intact basement membrane. Bladder tissue whose urothelium was detached without seeding other cell lines had some areas with a intact urothelium with up to 15 layers and a intact basement membrane. Between 10% and 30% of the tissue pieces with T24 parental cells were covered with urothelial cells with one to a indefinable number of layers with a intact basement membrane. In one sample was an area that looked like there were cells invading form the urothelium through the basement membrane, but certain determination was not possible. Between 10% and 20% of the tissue pieces with T24 control cells were covered with urothelial cells that form one to 15 layers and had a intact basement membrane. Like with T24 parental cells here are areas with potentially invading cells, but there is no proof. Between 20% and 50% of the tissue pieces with T24 GRHL3 cells were covered with urothelial cells that form one to 20 layers. In some areas no clear basement membrane was visible and it looked like there were cells invading from the urothelium, but certain determination was not possible.

A general observation were cell clusters beneath the urothelium or beneath der surface whose origin were unknown. As a result of this experiment, we have to state that T24 in this organoid model is possibly invasive. But a clear tendency towards a oncogenic or tumour suppressive effect of GRHL3 is not given. We also cannot determine if the cells we see are porcine cells or our cell lines.

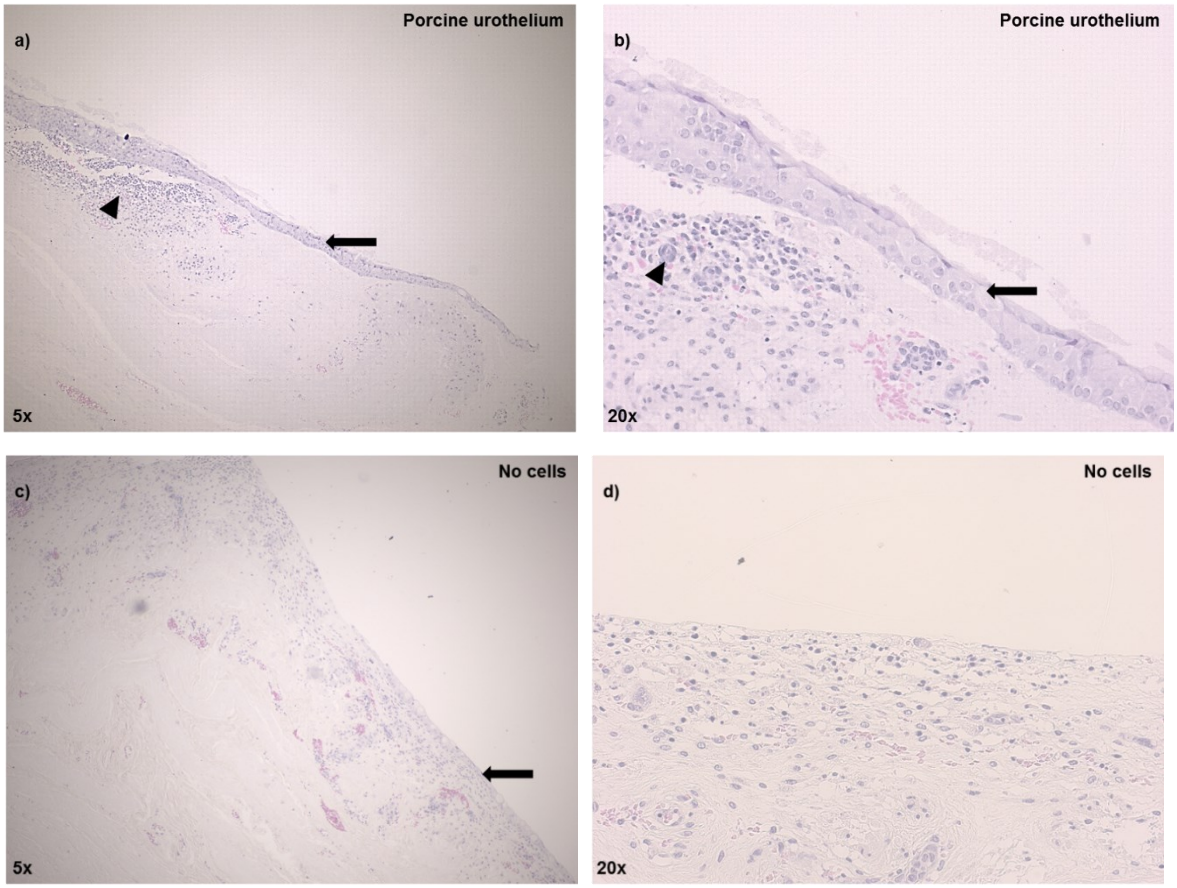


Figure 7 Controls of the porcine bladder organoid model. Porcine bladder without scrapping with a) 5x magnification and b) 20x magnification. Intact urothelium is visible (arrow). Beneath the surface cells are visible (arrow heads), but further determination is not possible. In the samples with scrapping and without seeded cells areas with urothelium (arrow) and without urothelium are visible in c) 5x magnification. In d) 20x magnification the surface without cells is visible.

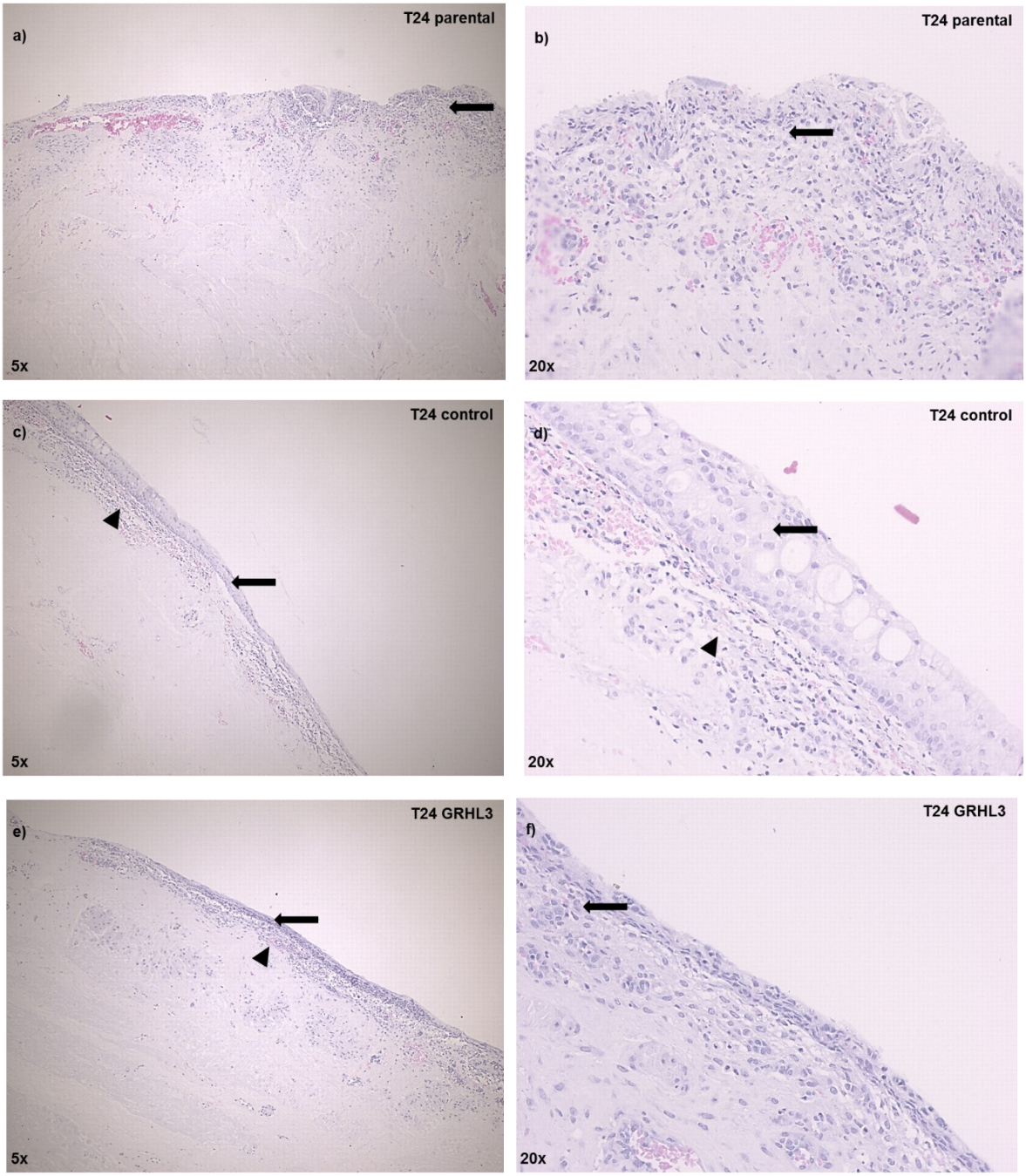


Figure 8 Invasion in the porcine bladder organoid model of T24. T24 parental may be invasive (arrows) in a) (5x magnification) and b) (20x magnification), but exact determination of the surface cells and the cells beneath the surface is not possible. T24 control forms a non-invasive layer (arrow) in c) 5x magnification and d) 20x magnification. Beneath the surface are cells of unknown origin (arrow heads). e) T24 GRHL3 form a non-invasive layer (arrow) with unknown cells (arrow head) beneath it (5x magnification). f) T24 GRHL3 could be growing invasive (arrow). There is no clear separation between the urothelial cells and the areas beneath it visible (20x magnification). GRHL3 = Grainyhead-like 3 transcription factor.

The two experiments that investigated invasion show no clear result. In the RTCA analysis *GRHL3* seems to have a oncogenic effect because it increases the invasiveness of T24. In the organoid model a general observation was the

invasiveness of T24. But a clear trend about the role of *GRHL3* is not given. Therefore, experiments about the migration potential were performed.

3.4 Investigation of the role of GRHL3 on the migration potential of bladder cancer cells

Migration of cells plays an important role in tumours. Because of that, the wound healing assay was used to investigate the influence of *GRHL3* on cell migration. RT4 and HT1197 cells with deleted *GRHL3* and T24 cells with overexpression of *GRHL3* were observed.

The RT4 cells were observed for 96 hours. RT4 parental was observed five times with four technical replicates, RT4 control was observed six times with four technical replicates, RT4 shGRHL3-1 was observed five times with four technical replicates and RT4 shGRHL3-2 was observed eleven times with four technical replicates. After this time the vacant area was not overgrown completely. Interestingly, RT4 control and RT4 shGRHL3-2 migrated slower than RT4 parental. RT4 shGRHL3-1 migrated faster than RT4 parental. The cells with the slowest migration were RT4 control. This could mean that the knockdown of *GRHL3* increases migration. But all in all, there was only one statistical significant result after 72 hours between RT4 control and RT4 shGRHL3-1. At the other time points was no statistically significant difference (Figure 9).

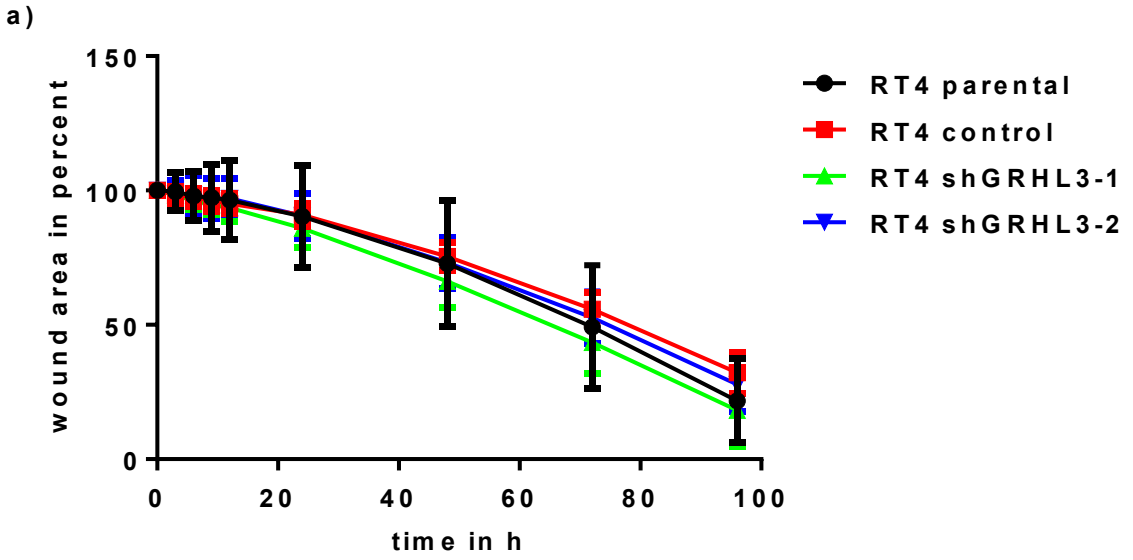


Figure 9 Migration of RT4 in the wound healing assay. The cells were observed for 96 hours. After this time, the gap was still not closed. The slowest cell line was RT4 control. But the only statistically significant result was after 72 hours between RT4 control and RT4 shGRHL3-1. GRHL3 = Grainyhead-like 3 transcription factor. H = hours.

The HT1197 cells were observed for 48 hours. After this time the uncovered area was overgrown. HT1197 parental was observed four times with four technical replicates, HT1197 control was observed three times with four technical replicates, HT1197 shGRHL3-1 was observed four times with four technical replicates and HT1197 shGRHL3-2 was observed four times with four technical replicates. HT1197 parental and HT1197 shGRHL3-2 migrated slower than HT1197 control and HT1197 shGRHL3-1. HT1197 shGRHL3-1 migrated slightly faster than HT1197 control. But in this experiment were no statistically significant results (Figure 10).

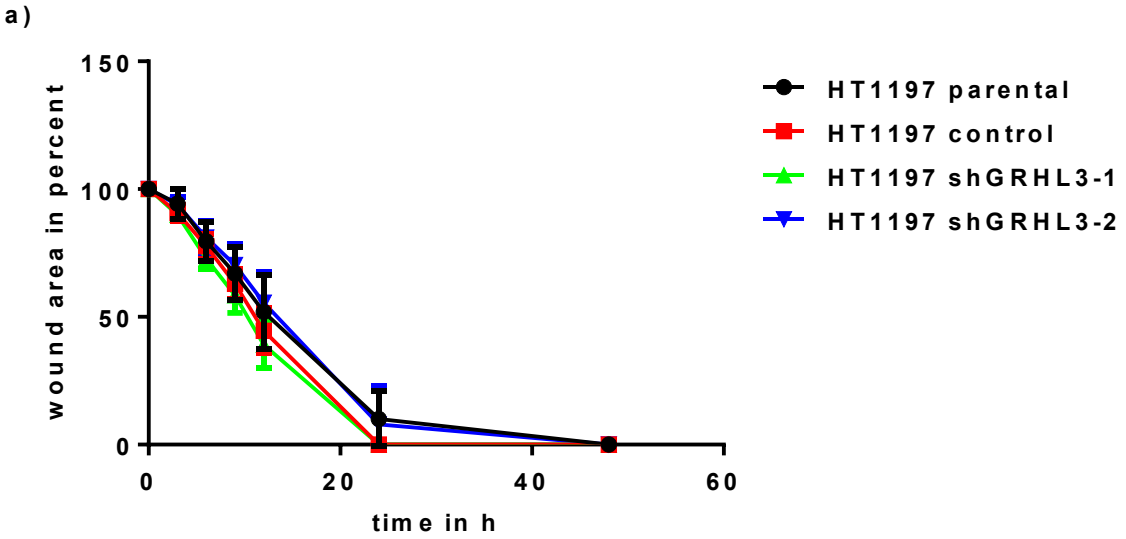


Figure 10 Migration of HT1197 in the wound healing assay. The cells were observed for 48 hours. The fastest cell line was HT1197 shGRHL3-1. HT1197 shGRHL3-2 migrated slower than HT1197 control. The results are not statistically significant. GRHL3 = Grainyhead-like 3 transcription factor. H = hours. Sh = short hairpin.

The T24 cells were observed for 24 hours. After this time the uncovered area was closed. The experiment was done five times with four technical replicates with T24 parental and six times with four technical replicates with T24 control and T24 GRHL3. The fastest cell line was T24 GRHL3. It was significantly faster than T24 control after 3 hours, 6 hours, 9 hours and 12 hours. Between T24 parental and T24 control were no significant differences (Figure 11). In T24 the influence of *GRHL3* on migration is consistent with the invasion in the RTCA analysis. *GRHL3* seems to have a oncogenic effect on the cells that do not express *GRHL3*. In HT1197 with a moderate expression, the migration is not altered.

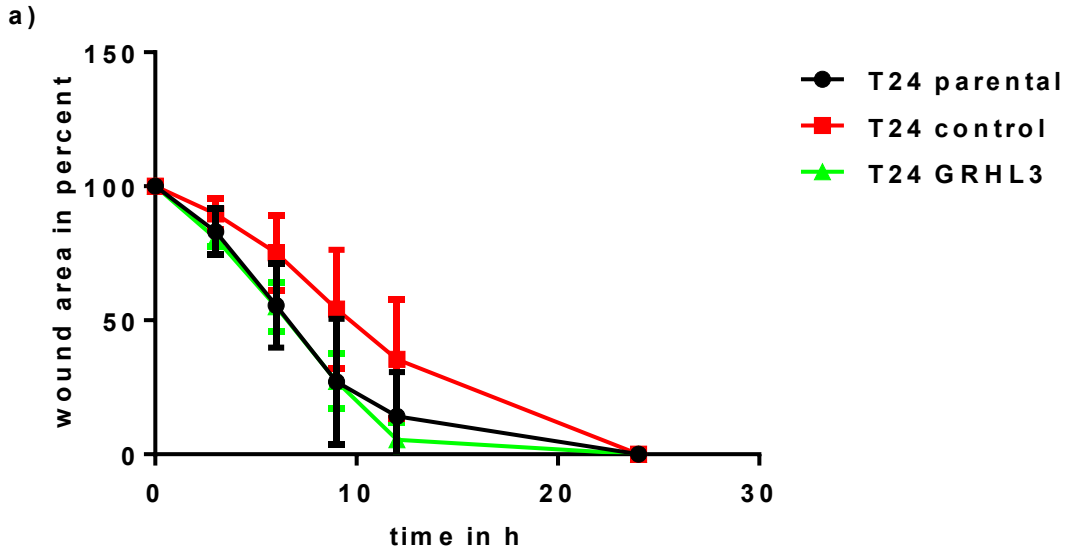


Figure 11 Migration of T24 in the Wound healing assay. The cells were observed for 24 hours. The fastest cell line was T24 GRHL3. It was significantly faster than T24 control after 3,6,9,12 h. P-values T24 control vs. T24 GRHL3: 3h: 0,0055; 6h: 0,0139; 9h: 0,0222; 12h: 0,0099. GRHL3 = Grainyhead-like 3 transcription factor. H = hours.

3.5 mRNA expression of cell cycle proteins and Uroplakins in bladder cancer cell lines

After performing functional assays, we were interested in the regulatory function of GRHL3 on a molecular level. Therefore, we investigated how the mRNA expression of differentiation markers and signal transduction proteins was affected by GRHL3. For this purpose, q-RT-PCR was used to investigate the influence of GRHL3 in T24.

All Uroplakins were investigated in one experiment with a triplicate. *UPIa* showed no significant difference in mRNA levels between the cell lines and the cells have nearly no expression (Figure 12a). *UPIb* had no expression in all cell lines in one experiment in triplicate (data not shown). *UPII* was statistically significant upregulated in T24 control compared to T24 GRHL3, but the mRNA levels of T24 GRHL3 were the same as of T24 parental. The expression was verry small in alle three cell lines (Figure 12b). *UPIIIa* was statistically significant upregulated in T24 control, but T24 GRHL3 showed no significant change compared to T24 parental. Also, in T24 GRHL3 it was less expressed than in T24 control (Figure 12c). *UPIIIb* was upregulated in T24 GRHL3 compared to T24 control, but the standard

deviation was the same range than the total mRAN level and the difference was not statistically significant (Figure 12d). When you look into these results, you may notice that the mRNA expression of Uroplakins is relatively small in all cell lines. Furthermore, the transfection with a plasmid seems to influence the expression because in T24 control the mRNA levels are the highest, except for UpIIIb. If you only compare the transfected cells T24 control and T24 GRHL3 and only look into the statistical significant results, GRHL3 expression seems to reduce the expression of Uroplakins and thus the differentiation of the cells.

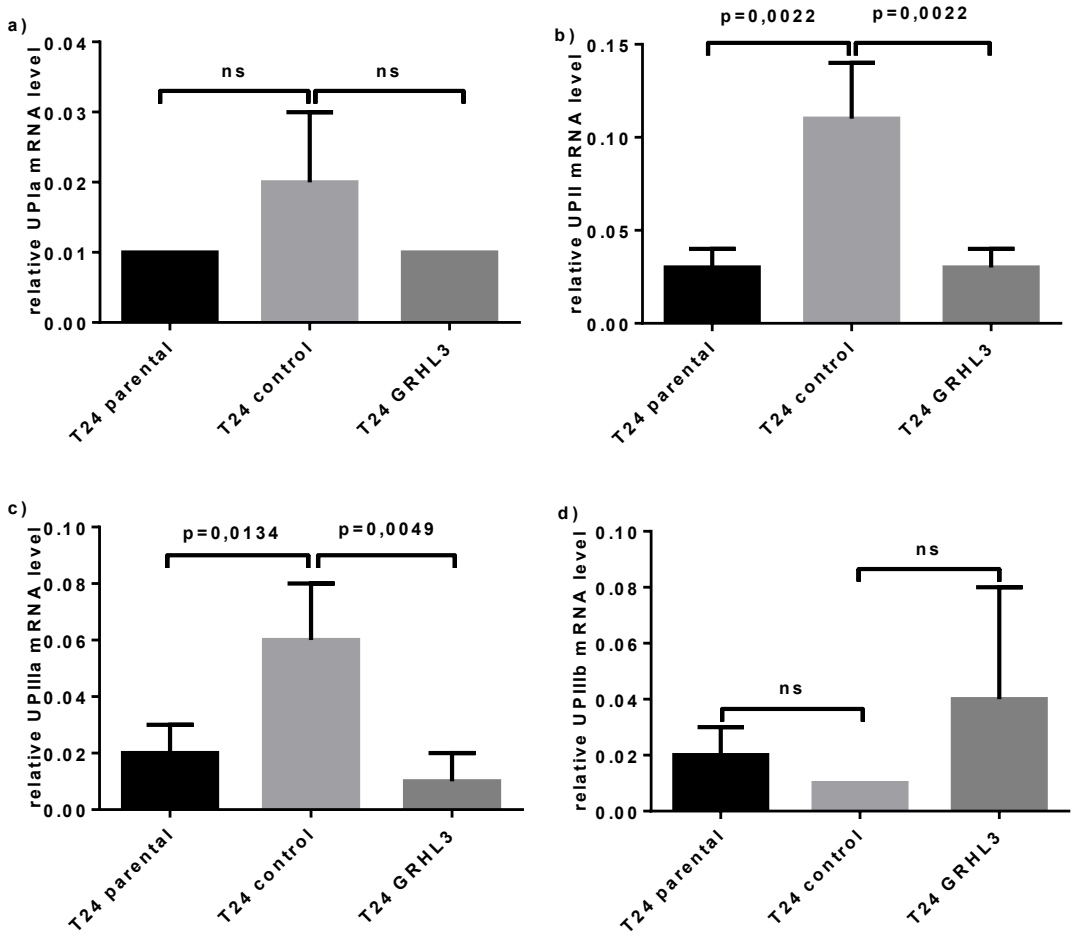


Figure 12 Messenger RNA (mRNA) expression of the differentiation markers, the Uroplakins, in T24. All experiments were done once in a triplicate. a) For Uroplakin Ia (UPIa) no significant change in expression was shown. b), c) Uroplakin II (UPII) and Uroplakin IIIa (UPIIIa) were significantly lower expressed in T24 GRHL3 than in T24 control. d) For Uroplakin IIIb (UPIIIb) were no significant differences seen. GRHL3 = Grainyhead-like 3 transcription factor. Ns = not significant.

After these results, we looked into the cell cycle protein PTEN. The following q-RT-PCR were performed once in a triplicate. *PTEN* was statistically significant

downregulated in T24 control and T24 GRHL3 compared to the parental T24 cells, but between T24 control and T24 GRHL3 was no statistically significant difference (Figure 13).

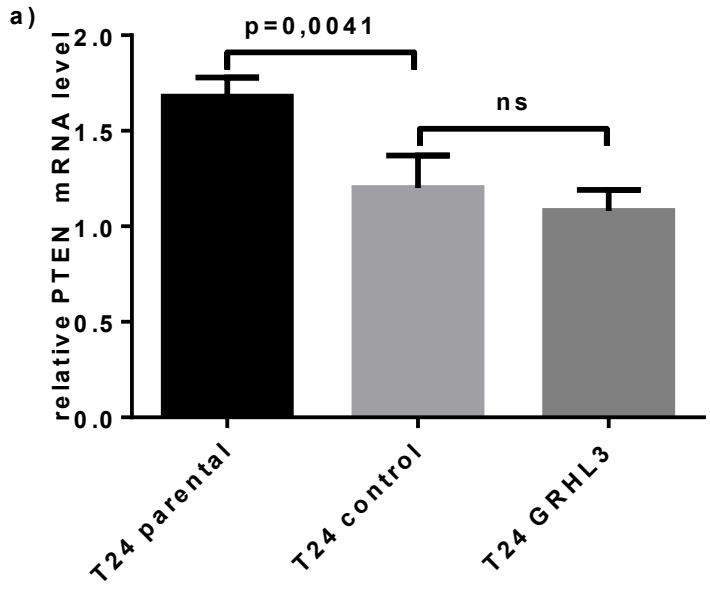


Figure 13 Messenger RNA (mRNA) levels of cell cycle protein Phosphatase and tensin homolog (PTEN) in T24. All experiments were performed one time in a triplicate. a) transfection itself has an influence in the expression of PTEN. When the cell lines with transfection, T24 control and T24 GRHL3, are compared, there are no significant differences in the expression of these genes. GRHL3 = Grainyhead-like 3 transcription factor. Ns = not significant.

3.6 Protein expression in parental and transduced bladder cancer cells

After investigating mRNA expression of several genes, we were now interested in the protein expression of signal transduction proteins that play a role for example in cell growth. Western Blots were used to investigate the influence of GRHL3 on different signal transduction proteins in T24 and RT4. Western Blots were performed. The signal intensity of the bands of the proteins of interest were compared to the signal intensity of the β-actin band. Then these relative protein expressions were compared to the expressions in the other cell lines.

The proto-oncogene AKT1 was investigated in three Western Blots in T24 and showed an increased expression in T24 control and T24 GRHL3 compared to T24 parental, but both not significant (Figure 14). In RT4 (one Western Blot) the

expression was ambivalent. In RT4 shGRHL3-1 AKT1 levels were increased, whereas the AKT1 levels in RT4 shRHL3-2 were decreased (Figure 15). Since only one experiment was performed, statistical analysis was not possible.

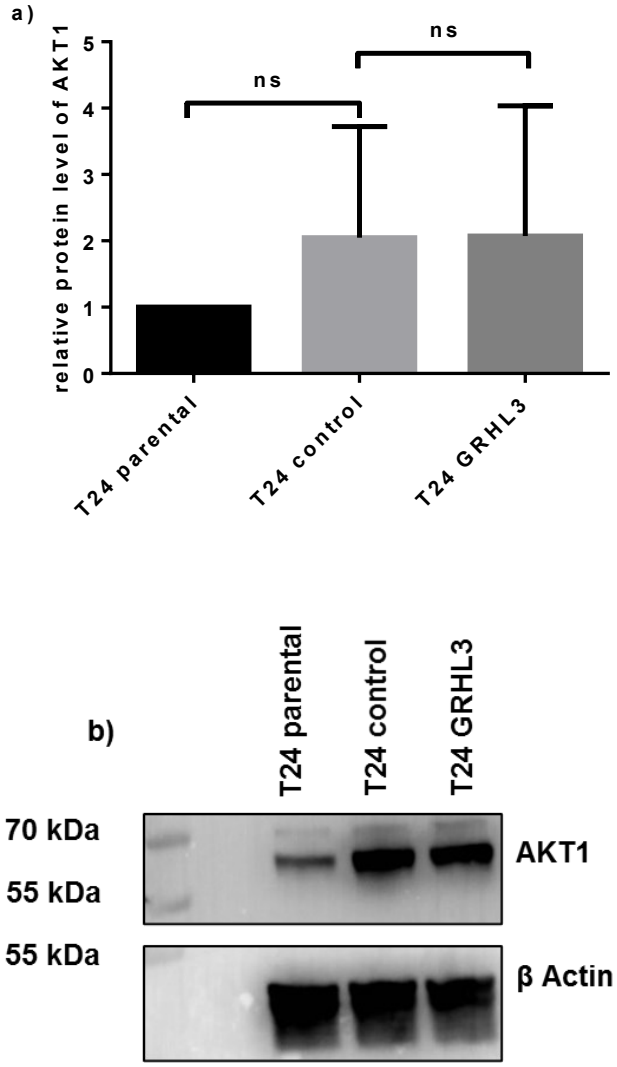


Figure 14 Protein expression in Western Blots of AKT1 in T24. a) Shown are an exemplary Western Blot and b) the quantification of AKT1 in T24. Three Western Blots were performed. AKT1 does not change significantly due to ectopic GRHL3 expression. GRHL3 = Grainyhead-like 3 transcription factor. Ns = not significant. kDa = kilo Dalton.

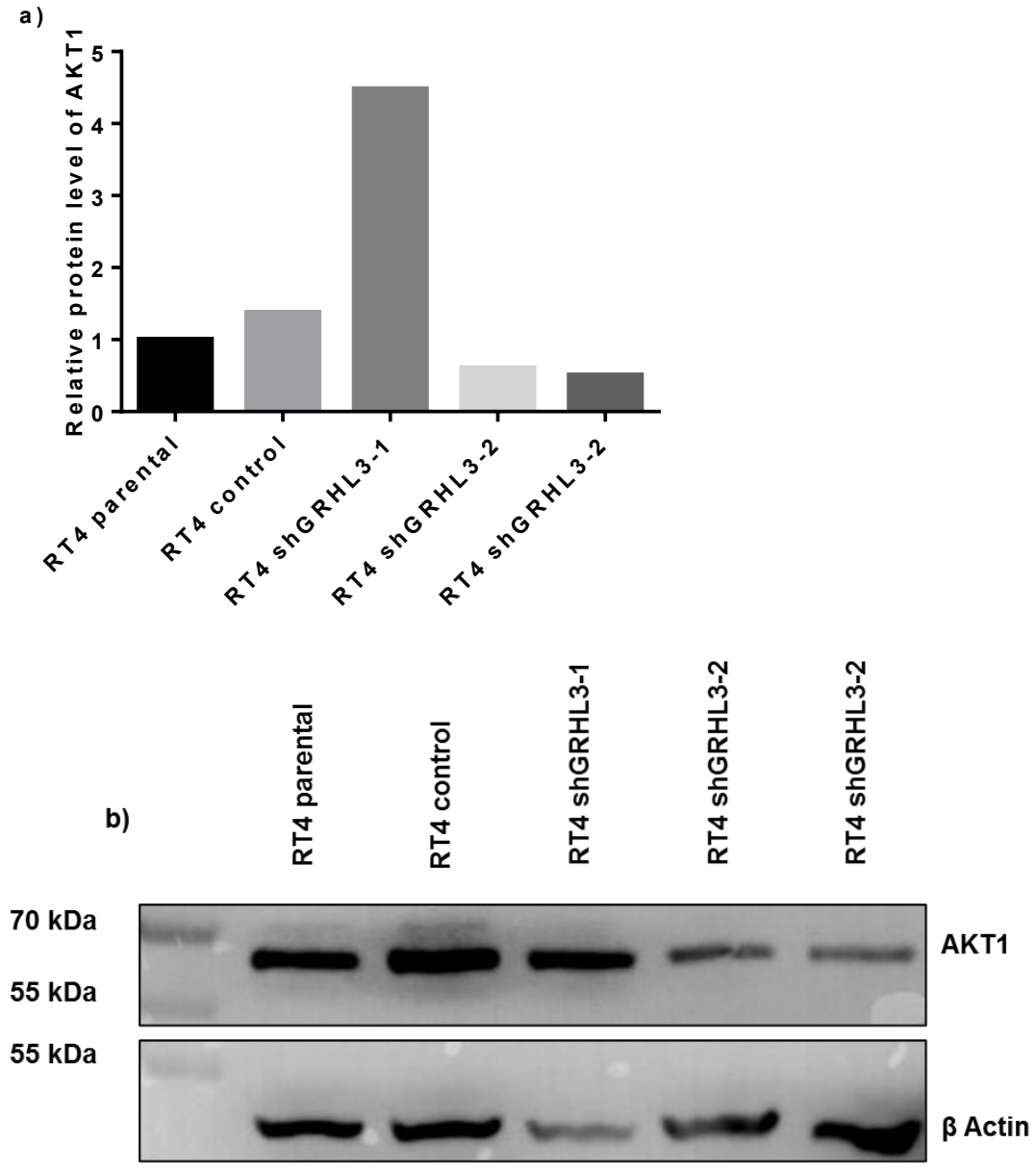


Figure 15 Protein expression in Western Blots of AKT1 in RT4. a) The only Western Blot and b) the quantification of AKT1 in RT4 are shown. AKT1 expression increases in the cell line transfected with shGRHL3-1 and decreases in the cell line transfected with shGRHL3-2. GRHL3 = Grainyhead-like 3 transcription factor. Sh = short hairpin. kDa = kilo Dalton.

Both pAKT(Ser473) and pAKT(Thr308) were investigated in three Western Blots in T24. PAKT1(Ser473) showed an increased expression in T24 GRHL3, but not significant (Figure 16). PAKT1(Thr308) showed a significant decreased expression in T24 control and T24 GRHL3 compared to T24 parental, but between T24 control and T24 GRHL3 are no significant differences (Figure 17).

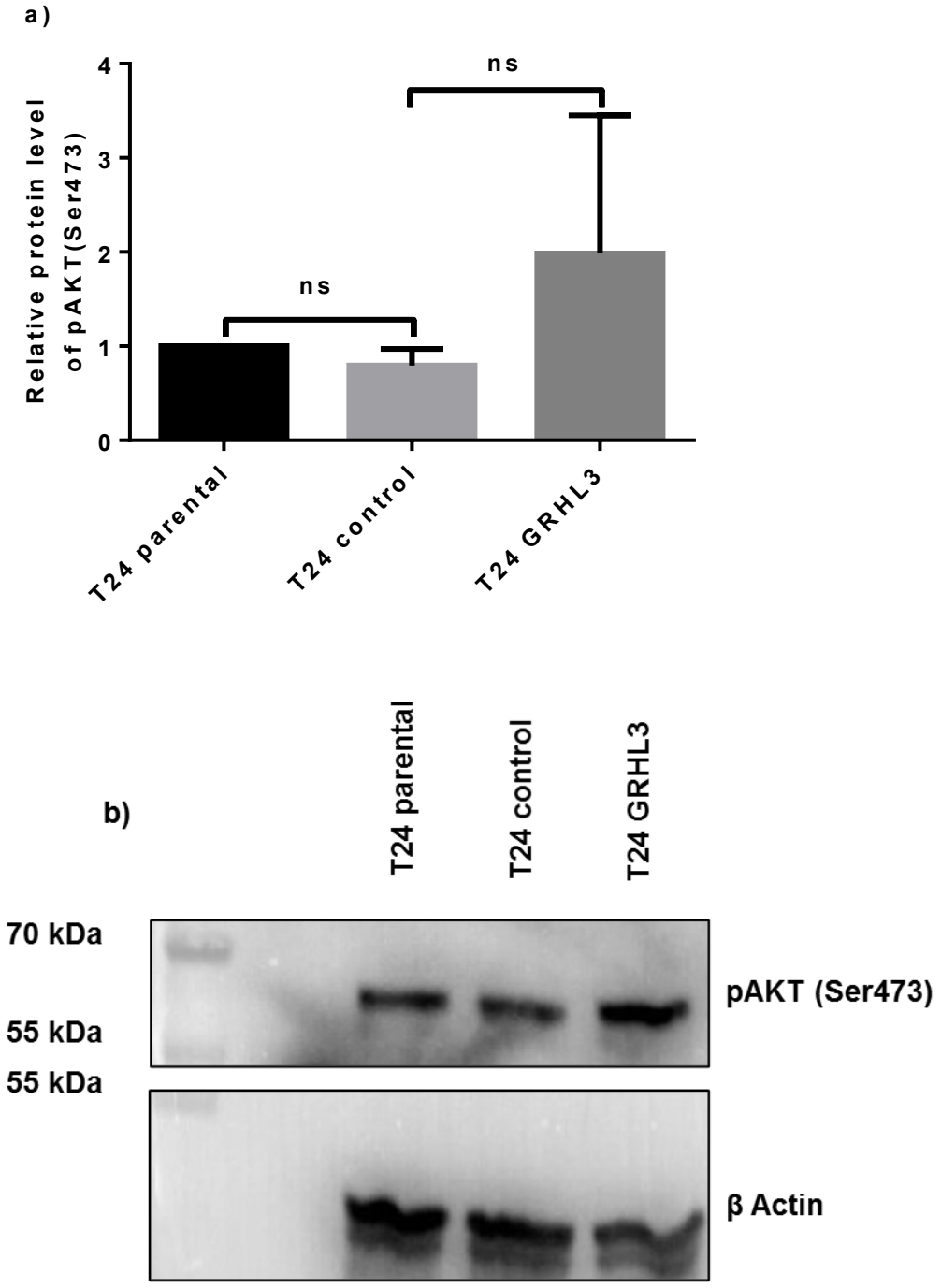


Figure 16 Protein expression in Western Blots of pAKT(Ser473) in T24. Shown are a) the quantification of the protein and b) an exemplary Western Blot. Three Western Blots were performed. pAKT(Ser473) is higher expressed in T24 GRHL3 than in T24 parental and T24 control but not significant. GRHL3 = Grainyhead-like 3 transcription factor. kDa = kilo Dalton. Ns = not significant.

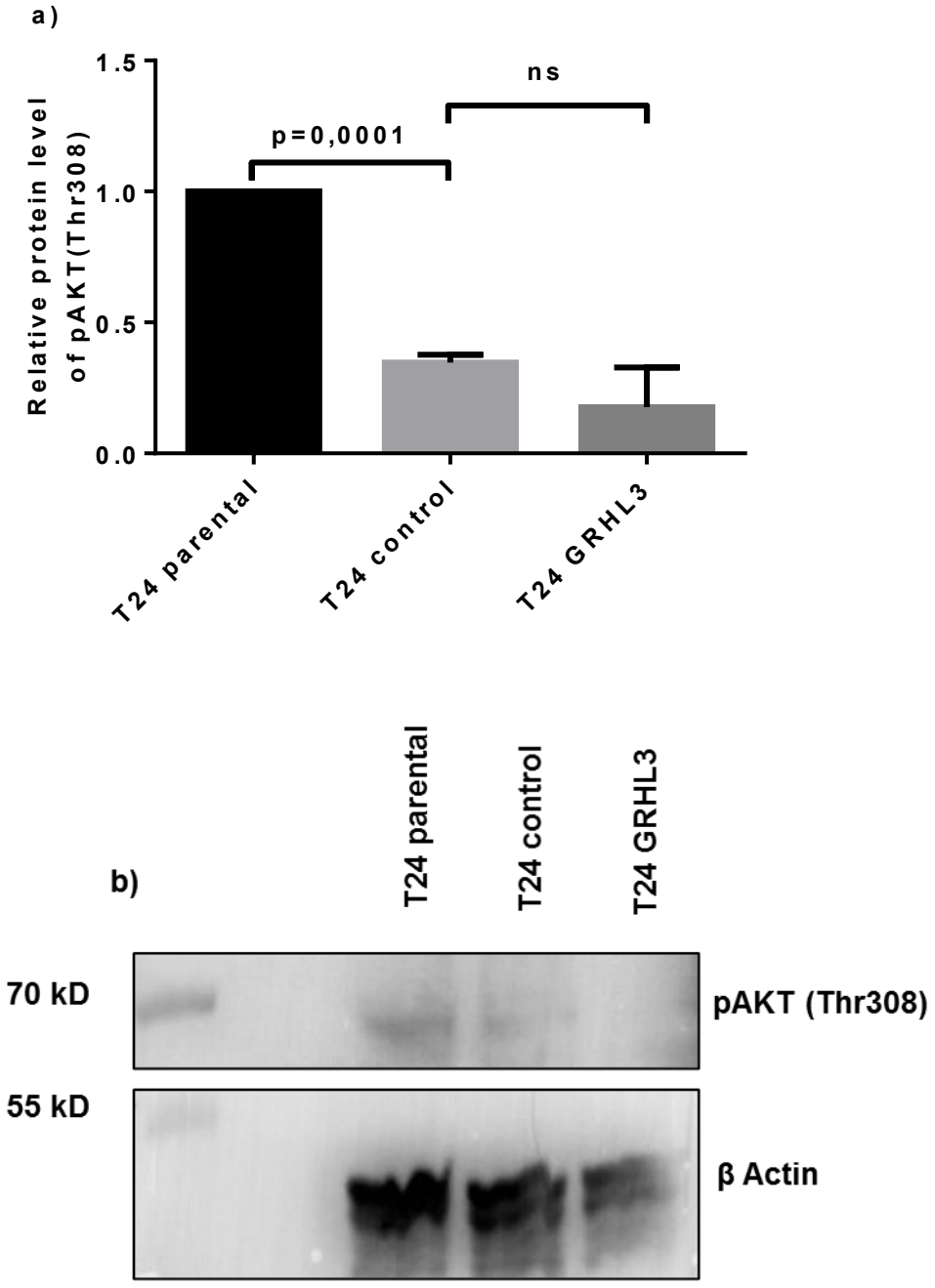


Figure 17 Protein expression in Western Blots of pAKT(Thr308) in T24. Shown are a) the quantification of the protein and b) an exemplary Western Blot. Three Western Blots were performed. pAKT(Thr308) is significantly lower expressed in T24 control and T24 GRHL3 than in T24 parental. Between T24 control and T24 GRHL3 are no significant differences. GRHL3 = Grainyhead-like 3 transcription factor. kD = kilo Dalton. Ns = not significant.

The proto-oncogene ERK1/2 was investigated in six Western Blots and showed no change in the protein expression in T24 (Figure 18). In RT4 ERK1/2 was investigated twice. Compared to RT4 control, ERK1/2 was slightly decreased in RT4 shGRHL3-1 and RT4 shGRHL3-2. All three were increased compared to RT4

parental (Figure 19). Phosphorylated ERK1/2 was investigated in three experiments in T24. No changes in T24 were observed (Figure 20).

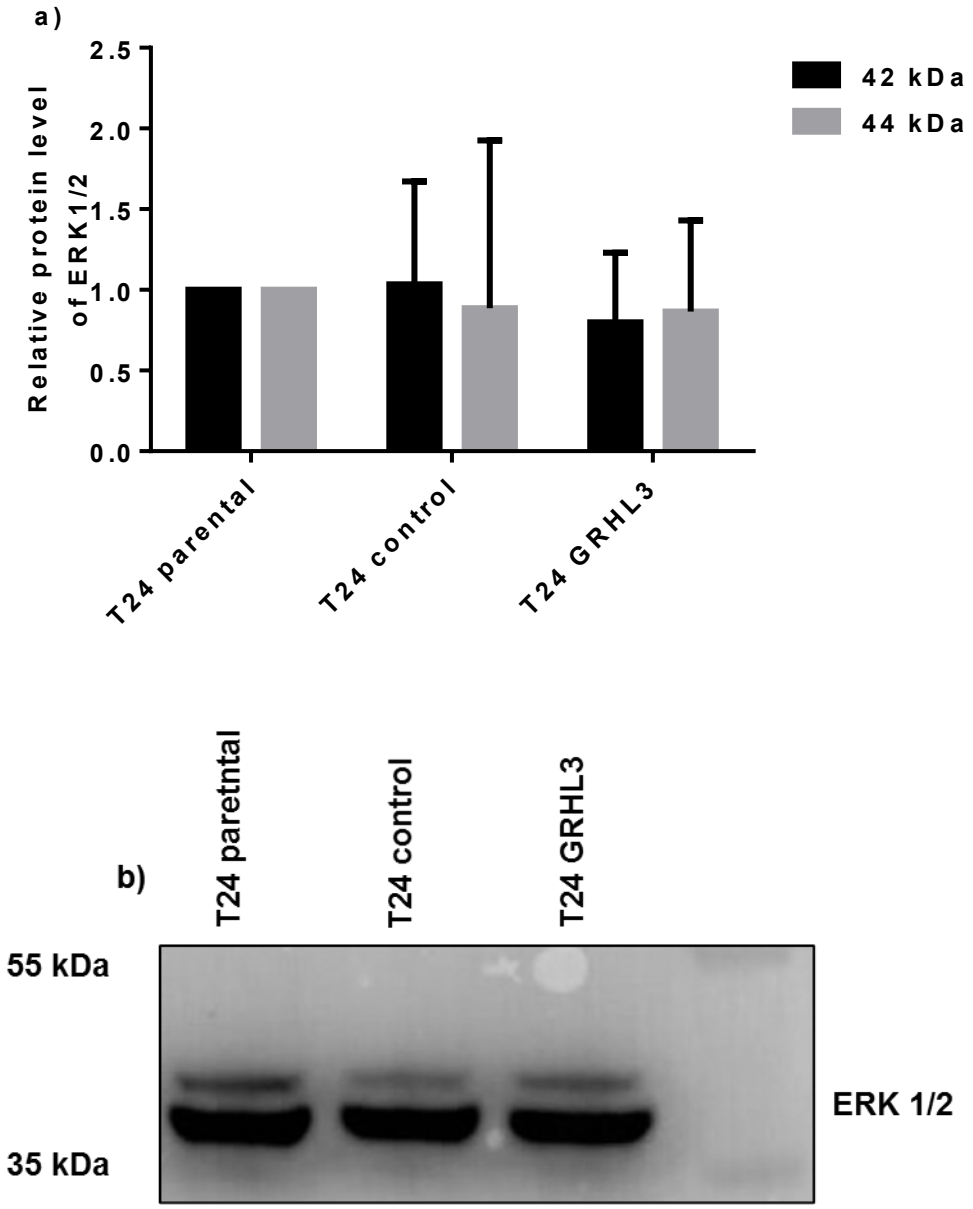


Figure 18 Protein expression in six Western Blots of Extracellular signal-regulated kinases 1 and 2 (ERK1/2) in T24. Shown are a) the quantification of the protein and b) an exemplary Western Blot. ERK1/2 expression was not altered in T24 in six Western Blots. GRHL3 = Grainyhead-like 3 transcription factor. kDa = kilo Dalton.

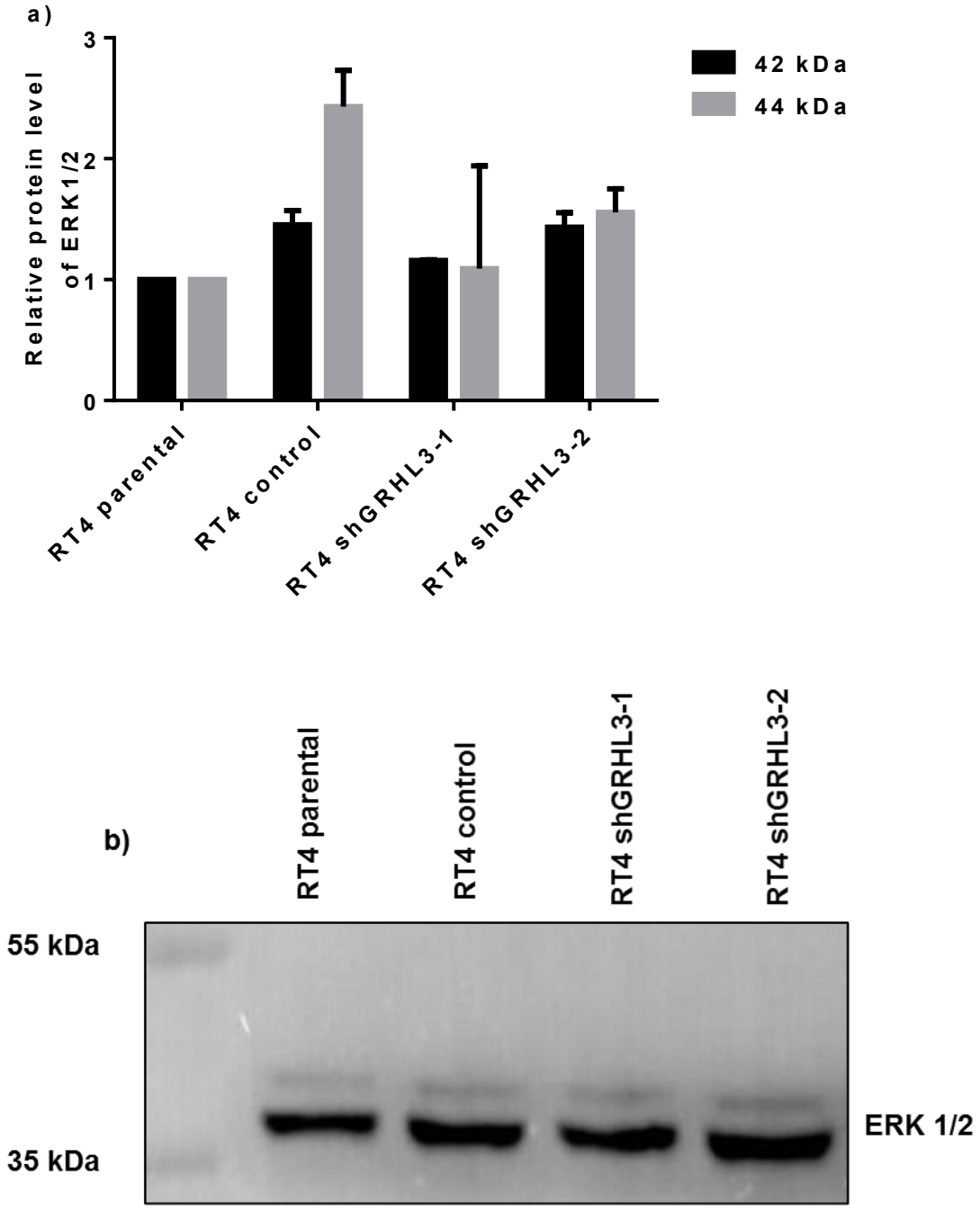


Figure 19 Protein expression in two Western Blots of Extracellular signal-regulated kinases 1 and 2 (ERK1/2) in RT4. Shown are a) the quantification of the protein and b) an exemplary Western. Two Western Blots were performed. Transfected cells show higher protein expression than RT4 parental. In RT4 shGRHL3-1 and RT4 shGRHL3-2 the protein levels are decreased compared to RT4 control. GRHL3 = Grainyhead-like 3 transcription factor. kDa = kilo Dalton. Sh = short hairpin.

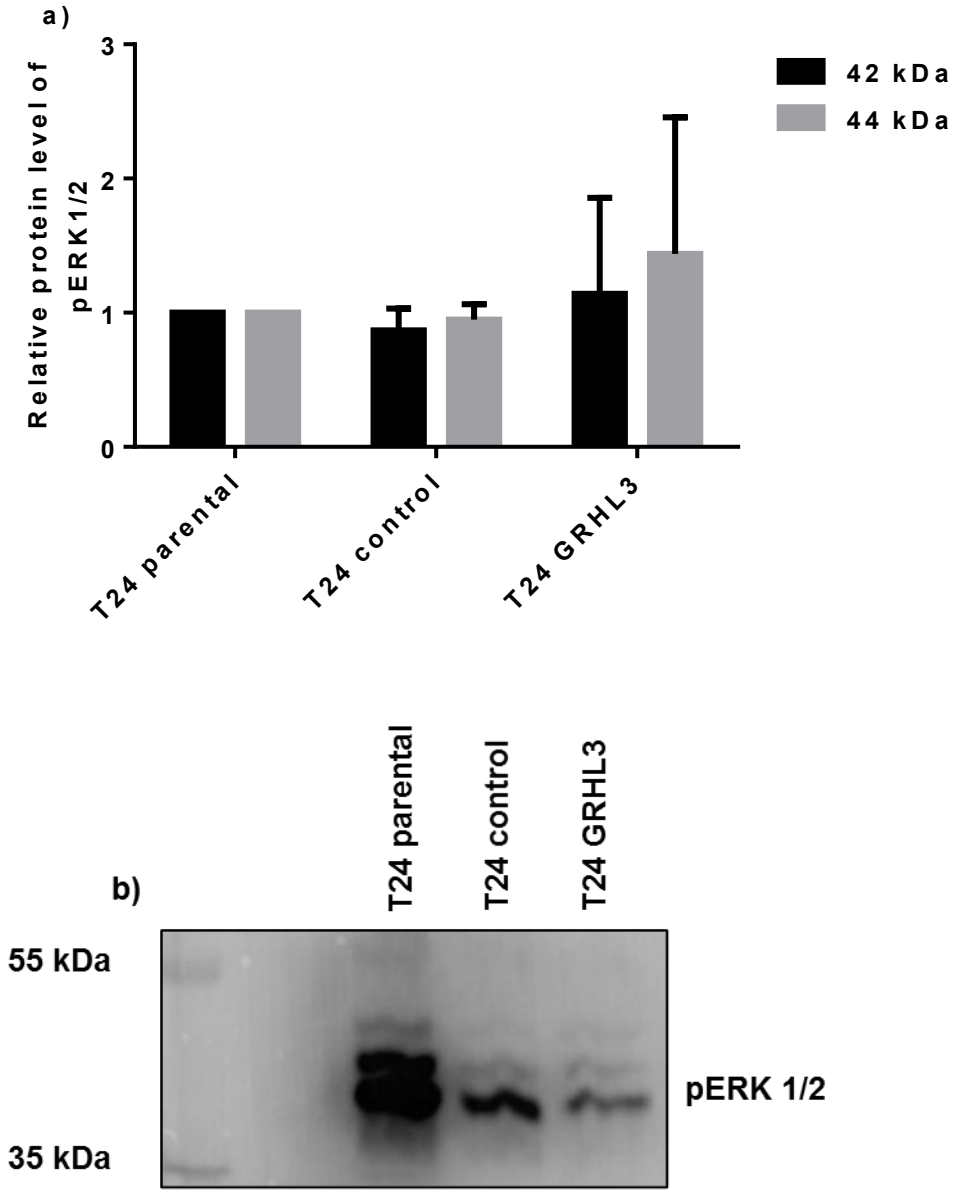


Figure 20 Protein expression in three Western Blots of phosphorylated Extracellular signal-regulated kinases 1 and 2 (pERK1/2) in T24. Shown are a) the quantification of the protein and b) an exemplary Western Blot. In three Western Blots the protein expression of pERK1/2 in T24 was not altered. GRHL3 = Grainyhead-like 3 transcription factor. kDa = kilo Dalton.

The tumour suppressor gene PTEN showed no significant changes in T24. PTEN was non-significantly increased in T24 GRHL3 compared to T24 control (Figure 21).

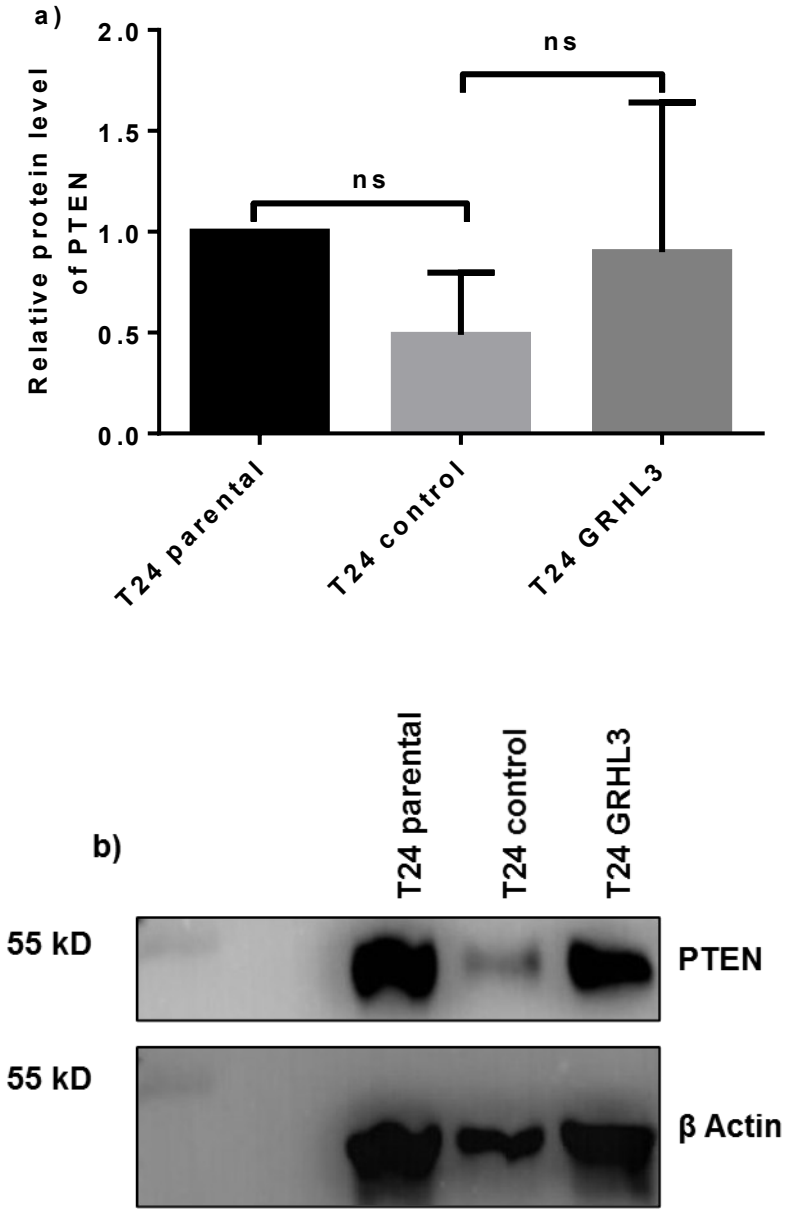


Figure 21 Protein expression of Phosphatase and tensin homolog (PTEN) in T24 in three Western Blots. Shown are a) the quantification of the protein and b) an exemplary Western Blot. No statistically significant alterations of the PTEN expression were observed in T24. In T24 GRHL3 PTEN expression is higher than in T24 control but not significant. GRHL3 = Grainyhead-like 3 transcription factor. kD = kilo Dalton. Ns = not significant.

Comparing all Western Blots, one could conclude that *GRHL3* does not alter the expression of the investigated proteins. *PTEN* was of special interest because it is described as a direct target of GRHL3. Although the expression increases in T24 when *GRHL3* was ectopically expressed compared to the control cells, no significancy was reached.

4. Discussion

Since the effect of *GRHL3* on epithelia formation and on different cancers was discovered, the question was raised if there is an effect on urothelial cancer, especially because this effect was not investigated before. Also as mentioned above *GRHL3* is important for correct urothelial formation in mice. To investigate these effects on human urothelial cancer different human cell lines were used and *GRHL3* was either ectopically expressed or knocked down. Unfortunately, the presented results are ambivalent.

At first, *GRHL3* mRNA expression levels between RT4, T24, HT1197 and UMUC3 were compared. The well differentiated and non-invasive RT4 cells show the greatest expression, the cell lines T24 and UMUC3, both from invasive cancers, have no expression at all. Interestingly, the invasive cell line HT1197 has a moderate expression of *GRHL3*. This indicates that *GRHL3* has a tumour suppressive effect. But the expression in HT1197 does not fit in. One explanation could be that a certain amount is necessary. Another explanation would be hat *GRHL3* is not solely important for tumourigenesis. In addition, GRHL3 is a upstream regulator of *PTEN*. [21] Downstream mutations of GRHL3, for example in the PTEN pathway, could explain why HT1197 is invasive despite expressing *GRHL3*.

To investigate the role of *GRHL3* the invasiveness was observed. Interestingly, invasive T24 cells became more invasive when *GRHL3* was overexpressed. This was shown with the Continuous Monitoring of Cell Impedance. In non-invasive RT4 cells a knock-down of *GRHL3* had no effect of the invasiveness, indicating that *GRHL3* alone is not responsible, but it could possibly increase the invasiveness of already invasive cells. As previous studies showed, GRHL3 cannot activate gene expression alone. Other transcription factors are required for this process. [27, 43] There could be another transcription factor that is expressed anyways and binds an enhancer after GRHL3 opens it and thus increases the invasiveness of T24.

In the porcine bladder organoid model only one cell line, T24, could be observed. Independent of the expression of GRHL3 these cells seem to be invasive. But

there are some problems about this experiment. Even in the controls that were scrapped are some urothelial cells visible. Therefore it is not really possible to distinguish if we see the seeded cells or normal urothelial cells in this experiment. Also there are cells beneath the surface whose origin is not known. They could be leukocytes, but this question remains unclear. Furthermore it was not possible to differentiate if the cancer cells are invasive or not because we did not stain the basement membrane and we did not specifically stain the T24 cells. So further work is necessary. On one hand staining of the basement membrane is necessary. On the other hand specific staining of T24 is necessary to distinguish T24 from normal urothelial cells and to see if T24 is invasive or forms micro metastases. Our working group was able to solve this problem after these experiments were conducted. A HLA-antibody was used to stain T24 immunohistochemically and distinguish them from the porcine tissue. [85] The organ culture model could be used in future experiments to investigate the invasiveness of different cell lines. Compared to pure in-vitro experiments this model allows the interaction of cell lines with healthy tissue.

With the migration assay the cell lines RT4, T24 and HT1197 were observed. The results were ambivalent. The results of RT4 were similar to invasion. RT4 did not migrate different when *GRHL3* was knocked down. In HT1197 with a moderate *GRHL3* mRNA expression migration was not altered after the knock-down. T24, however, changed after the expression of *GRHL3*. It migrated significantly faster, supporting the results observed in invasion. Perhaps the same mechanism we suppose in invasion is responsible: expression of *GRHL3* opens enhancers for other transcription factors that are expressed anyways and thus increases migration.

As mentioned above studies showed that at least Uroplakin *UpII* is a direct target of GRHL3 in mice and *GRHL3* is important for urothelial differentiation. [91] These results, however, show that *GRHL3* has a reverse effect on Uroplakins. *UPII* and *UPIIIa* have a lower mRNA expression in *GRHL3* transfected cells compared to the control. It is important to mention that the introduction of a plasmid alone alters the expression of Uroplakins and the expression in both cells is quite marginal. But when only the transfected cells are compared, *GRHL3* expression represses the expression of Uroplakins and therefore differentiation. In addition, it was shown

that in advanced tumour stages differentiation associated expression of Uroplakins is lost and even metastases express Uroplakins. [65] Therefore, the expression respectively missing expression of Uroplakins in T24 is not necessary a sign of differentiation. But FOXA1 is known as another differentiation marker, especially in luminal bladder cancer. [18, 46] After the presented experiments were conducted, our working group showed that GRHL3 increases the expression of *FOXA1* in T24 that lacks FOXA1, at least in the organ culture model, and has no effect on Uroplakins in vitro. This indicates that for differentiation the interaction with tissue is necessary and the expression of Uroplakins could be investigated in the organ culture model. In RT4 that expresses *FOXA1* the knock down of *GRHL3* does not alter *FOXA1* expression in the organ model. [85]

PTEN is described as a target of GRHL. [21] Interestingly, our results cannot validate this. Expression of *GRHL3* in T24 did not lead to an increase of PTEN expression neither on mRNA levels nor on protein levels. Indeed, the protein expression was higher compared to T24 control but the difference was not significant. The downstream target AKT1 that is inhibited by PTEN was investigated, too. In Western Blot no significant alterations of AKT1 in T24 were shown. In RT4 the result was ambivalent. In RT4 shGRHL3-1 AKT1 was increased and in RT4 shGRHL3-2 AKT1 was decreased. Since only one Western Blot was performed, no statistical analysis was possible. The active forms of AKT1, pAKT(Ser473) and pAKT(Thr308), were not altered by GRHL3, too. The results indicate that the PTEN/AKT pathway may not be influenced by *GRHL3* in cancer, at least not in this model, although *PTEN* is described as a direct target of GRHL3.

Furthermore, we were interested in MAPK pathway that has a oncogenic role. Therefore Western Blots to measure the protein expression of the MAP-Kinase ERK were conducted. In T24 ERK expression did not change after expression of *GRHL3*. In RT4 the ERK expression decrease slightly after *GRHL3* knock down compared to RT4 control. Phosphorylated ERK was not altered after GRHL3 was expressed ectopically in T24.

Taken together *GRHL3* to increase the invasiveness and the migration potential in always invasive T24 cells. In non-invasive RT4 cells with endogenous *GRHL3* expression the knock down did not increase the invasiveness of the cells, indicating that GRHL3 acts as oncogene in urothelial bladder cancer. Furthermore,

on a molecular level the ectopic expression of *GRHL3* in T24 reduced the expression of Uroplakins. This implies that *GRHL3* impairs differentiation in already non-differentiated cells. But one has to state that the expression of Uroplakins was quite low and that *GRHL3* was previously described as very important for differentiation of healthy urothelium. In addition, our working group has showed that *GRHL3* increases differentiation in T24, as mentioned above. There was no major effect on pathway proteins due to *GRHL3* expression, especially not on *PTEN*, a direct target of GRHL3 in previous studies. Furthermore, the question is raised if the introduction of plasmids provides a good model for investigating the effects of different genes and proteins, because the introduction itself seems to influence the expression of different key proteins of signalling cascades.

Analyses have been made to investigate which gens are direct targets of GRHL proteins in healthy tissue and in different cancers. Interestingly only 2 of the 50 most regulated genes in healthy tissue are dysregulated in different cancers indicating that the GRHL-pathways are not the most important ones in cancers, although it plays an important role in development and homeostasis of different epithelia. [60] Further studies could aim to validate these analyses and investigate it especially for urothelial bladder cancer.

5. Summary

Bladder cancer is the ninth most common malignant disease. There are two known pathways leading to urothelial bladder cancer. One leads to papillary non-muscle invasive bladder cancer and one leads to muscle invasive bladder cancer via carcinoma in situ. Most patients present with non-muscle invasive bladder cancer.

The protein Grainyhead-like protein 3 homolog (GRHL3) is important for correct development and differentiation of epithelia including the urothelium. In mice it was shown that the expression of Uroplakins, important for the differentiation of urothelial cells and the structure of the asymmetric unit membrane (AUM), and the expression of *GRHL3* correlate. In squamous cell cancer (SCC) and basal cell cancer (BCC), both cancers of the skin, GRHL3 suppresses the tumours. For other cancers like lung cancers studies showed an increased expression of *GRHL3* in advanced stages. The role of *GRHL3* in urothelial bladder cancer was not subject of studies up to now.

Therefore this study investigated the role of *GRHL3* in urothelial bladder cancer. Urothelial bladder cancer cell lines were used as model. GRHL3 was either overexpressed, knocked-down or knocked-out using lentiviral vectors. These cell lines were used for the migration assay. To investigate the effects of GRHL3 on invasion the Continuous Monitoring of Cell Impedance and a porcine bladder organoid model were used. The effect on the messenger RNA (mRNA) levels of Uroplakins and Phosphatase and tensin homolog (PTEN) were quantified using quantitative real time polymerase chain reaction (q-RT-PCR) and the effect on the protein levels of signal transduction proteins were investigated with Western Blots. Taken together this study investigated important aspects of cancer, in particular migration, invasion, differentiation and signal transduction.

The results for the invasion are not clear. GRHL3 seems to make already invasive cells (T24) more invasive, whereas in non-invasive cell lines no effect was shown. The porcine bladder organoid model showed no clear results, because it was not possible to distinguish between porcine urothelial cells, our cell lines and other cells beneath the surface of the bladder. The effects on migration are similar to

invasion, because in T24 GRHL3 made the cells migrate faster, in RT4 and HT1197 it did not change the migration potential. The effects investigated by q-RT-PCR and Western Blot remain unclear. On mRNA level, significant results were only shown for Uroplakins (differentiation markers) in T24. *GRHL3* seems to repress Uroplakins compared to the control cell line. But compared to the parental cell line, no differences were observed. Unlike previous studies, we could not prove that *PTEN* is a target of GRHL3.

Taken together the effects of *GRHL3* on urothelial bladder cancer remain unclear and further investigation is necessary.

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7. Curriculum vitae

The CV has been removed for privacy reasons.