Relaxin in human thyroid neoplasias

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For Bogusz, Jagoda and my parents

Zusammenfassung

Das Peptidhormon Relaxin war für lange Zeit als Reproduktionshormon im weiblichen Reproduktionstrakt angesehen. In letzter Zeit wurden neue Ziele für Relaxin identifiziert. Meine Arbeit zeigt den Nachweis von Relaxin und RXFP1 Rezeptor auf Transkriptions- und Proteinebene in benignen und malignen Schilddrüsengeweben. *Follikuläres Schilddrüsenkarzinom* (FTC), *Papilläres Schilddrüsenkarzinom* (PTC) und *Undifferenziertes Schilddrüsenkarzinom* (UTC) exprimieren Relaxin im Gegensatz zu Schilddrüsenadenom, Struma- und Basedow-Geweben. In allen getesteten Gewebeproben konnte RXFP1 (Transkript) nachgewiesen werden. Untersuchungen mit humanen Relaxin 2 auf stabil transfizierten Schilddrüsenkarzinomzellen (FTC-133 und FTC-238) zeigten eine erhöhte Fähigkeit zur Penetration von extrazellulärer Matrix. Die erhöhte Expression von Cysteinproteasen wie Cathepsin L und D, und Metalloproteasen (MT1-MMP, MMP-2, ADAM23 und ADAMTS-5) in den Relaxinklonen korrelierte mit der Fähigkeit dieser Zellen sowohl Elastin- als auch Kollagen-Matrix zu degradieren. Ebenso erhöhte Relaxin die Motilitätsrate von FTC-133 und FTC-238 Zellen. Relaxin induzierte Veränderungen der epithelialen Schilddrüsenzellen zu fibroblastischer Morphologie. Die Untersuchungen des Zytoskeletts zeigten Unterschiede in der Lokalisierung von F-Aktin. Weitere Analysen zeigten den Einfluss von Relaxin auf die Expression und posttranslationale Modifikation von Zytosklett-assoziierten Proteinen wie Cofilin. Durch *in vivo* Versuche an Nacktmäusen wurde eine erhörte Fähigkeit der transfizierten Zellen Tumoren zu induzieren nachgewiesen.
Die Ergebnisse dieser Arbeit ziegten, dass Relaxin ein Modulator von *in vivo* Tumorwachstum und *in vitro* Invasivität von Schilddrüsentumorzellen ist.

Abstract

The relaxin hormone was for the long time considered as reproductive hormone in females. Recently new targets for relaxin peptide were identified. My studies investigated the expression of relaxin and RXFP1 receptor on transcript and protein level in benign and malignant thyroid tissues. Relaxin was found in follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC) and undifferentiated thyroid carcinoma (UTC) but not in adenoma, goiter and Graves' tissues. The relaxin receptor RXFP1 (mRNA) was detected in all samples tested. Examinations of follicular thyroid carcinoma cells (FTC-133 and FTC-238) stably transfected with human relaxin 2 revealed their increased ability to penetrate extracellular matrix (ECM). Increased protein level of cysteine proteases like Cathepsin L and D, as well as metalloproteases (MT1-MMP, MMP-2, ADAM23 and ADAMTS-5) in relaxin-expressing cells coincided with higher ability of these cells to degrade elastin and collagens matrix. Furthermore, relaxin elevated the motility rate of FTC-133 and FTC-238 cells. Relaxin induced morphological alternations of epithelial thyroid cells leading to fibroblast-like shape. Analysis of cytoskeletal proteins revealed differences in F-actin (fibrilar) localisation. Further investigations displayed influence of relaxin on expression and post-translational modifications of cytoskeleton-associated proteins like cofilin. Investigations *in vivo* performed on nude mice with xenotransplanted transfectants provided further evidence about the tumour promoting character of relaxin in thyroid carcinoma cells.

Our data indicated that in thyroid carcinoma cells relaxin is modulator of tumour growth *in vivo* and invasiveness *in vitro*.

Abbreviations

293T	Fetal kidney cell line
8305 C	Undifferentiated thyroid carcinoma cell line
8505 C	Undifferentiated thyroid carcinoma cell line
aa	Amino acid
Ab	Antibody
AC	Adenyl cyclase
ACN	Acetonitrile
ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with trombospondin motive
ADF	Actin depolymerising factor
ADP	Adenosine diphosphate
AG	Antigen
AJCC	The american joint committee on cancer
APS	Ammoniumpersulfate
AR	Androgen receptor
Arp2/3	Actin-related protein 2/3
ATP	Adenosine triphosphate
BBEC	Bovine bronchial epithelial cells
BCA	Bicinchoninic acid
BC-PAP	Papillary thyroid carcinoma cell line
BMP-1	Bone morphogenic protein-1
bp	Base pair
BrdU	Bromodeoxy uridine
BSA	Bovine serum albumine
C-643	Undifferentiated thyroid carcinoma cell line
Ca^{2+}	Calcium
cAMP	Cyclo-adenosine monophospate
cDNA	Complementary DNA
CF-33	Canine mammary carcinoma cells
cGMP	Cyclic GMP
CHAPS	(3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate)
CMV	Cytomegalovirus
CSL	Cranial suspensory ligament
DAB	Diaminobenzidine
DEPC	Diethylpyrocarbonat
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	2-deoxynucleoside 5'-triphosphates
DTT	Dithiothreitol
E. coli	Escherichia coli
ECL	Enhanced chemiluminiscence

ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epithelial growth factor receptor
ELISA	Enzyme-linked immuno sorbent assays
EMT	Epithelial-mesenchymal transition
Endo H	Endo-b-N-acetyl glucosainidase H
ER	Estrogen receptor
ERK	Extracellular-signal egulated kinase
EtOH	Ethanol
FA	Formaldehyde
F-actin	Fibrilar actin
FCS	Fetal calf serum
FSHR	Follicle stimulating hormone receptor
FTC	Follicular thyroid carcinoma
g	Gram
G-actin	Globular actin
GAM	Goat anti mouse
GAR	Goat anti rabbit
GDP	Guanosino diphosphoran
GPCR	G-protein coupled receptors
GTP	Guanosino triphosphoran
h	Hour
H2 relaxin	Human relaxin 2
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HBSS	Hank's balanced salts solution
HCl	Salt acid
HEC-1B	Human endometrial cancer cells
hECS	Human endometrial stromal cells
HPC-YP	Pancreatic carcinoma cell line
hRLN2	Human relaxin 2
HT1080	Fibrosarcoma cell line
HTh-74	Undifferentiated thyroid carcinoma cell line
IAA	Iodoacetamide
IBMX	3-isobutyl-1-methyl-xanthine
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Il-1β	Interleukin – 1β
lGF I	Insulin-like growth factor I
INSL3	Insulin-like peptide 3

INSL4	Insulin-like peptide 4
INSL5	Insulin-like peptide 5
INSL6	Insulin-like peptide 6
IR	Ischemia and reperfusion
IRES	In ribosome enter site
kDa	Kilo dalton
KLE	Endometrial carcinoma cells
KO	Knock-out
LAV	Large intracellular acidic vesicles
LB-A	Luria broth with ampicilin
LDLR	Low-density lipoprotein receptor-like
Ley-I-L	Leydig insulin-like peptide
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LIMK1/2	LIM kinase 1/2
M1R	Mouse relaxin 1
M6P	Mannose-6-phosphate
M6PR	Mannose-6-phosphate receptors
mA	Milliampere
MALDI-ToF	Matrix-assisted laser desorption ionization time of flight
MCA1	Relaxin monoclonal antibody
MCF-7	Breast cancer cell line
MDA-MB-231	Breast cancer cell line
MDCK	Maolin-Darby canine kidney
MEK	Map-ERK kinase
MetOH	Methanol
mg	Milligram
MHC	Major histocompatibility complex
min.	Minute
ml	Milliliter
MLCK	Myosin light chain kinase
mM	Millimol
mm^3	Cubic millimeter
MMP	Matrix metalloproteinases
MOCK	Negative control
mRNA	Messenger RNA
MS	Mass spectrometry
MTC	Medullary thyroid carcinoma
MT-MMP	Membrane type MMP
MTT	3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
$NADH_2$	Nicotinamide adenine dinucleotide
$NaHCO_3$	Natrium carbonate
ng	Nanogram

NH_2	Ammonium
NH_4Cl	Ammonium chloride
NH_4HCO_3	Ammonium hydrogen carbonate
NI	Nucleus incertus
nm	Nanometer
OD	Optical density
P1 relaxin	Porcine relaxin 1
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered-saline
PBS-T	Phosphate-buffered-saline - Tween
PC-3	Human prostate carcinoma cell line
PCR	Polymerase chain reaction
PDE	Phosphodiestrases
PDP	Progesterone-dependent protein
PFA	Paraformaldehyde
PgR	Progesterone receptor
PI3-K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol bisphosphate
PKA	Proteine kinase A
PKC	Proteine kinase C
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP2C	Protein phosphatase 2C
PTC	Papillary thyroid carcinoma
PTH	Parathyroid hormone
Rac	GTPase
rH2	Human relaxin 2
RHO	GDP dissociation inhibitor
RhoA	GTPase
RLF	Relaxin-like factor
RLN1	Relaxin 1
RLN2	Relaxin 2
RLN3	Relaxin 3
RNA	Ribonucleic acid
ROCK	RHO associated kinase
RT	Reverse transcription
RT-PCR	Reverse transcription- polymerase chain reaction
RXFP1	Relaxin Family Peptide Receptor 1
RXFP2	Relaxin Family Peptide Receptor 2
s	Second
SALPR	Somatostatin and angiotensin like peptide receptor
SDS	Sodium dodecylsulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

SF-1	Steroidogenic factor-1
si	Small interfering
SK-BR3	Breast cancer cell line
SSH	Slingshot
T3	Triiodotyronine
T4	Thyroxine
Taq	Thermus aquaticus.
TBE	TRIS-Boric acid-EDTA
TCA	Trichloro acetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TESK	Testicular protein kinase 1
TFA	Trifluoroacetic acid
TGF-β̃	Transforming growth factor – β
THP-1	Leukemia cell line
TIMP	Tissue inhibitor of metalloproteinases
T_M	Melting temperature
TM	Transmembrane
TNF-alpha	Tumour necrosis factor
TNM	Tumour-node-metastasis
Tris	Tris(hydroxymethyl)aminomethane
TSHR -	Thyroid stimulating hormone receptor
TWEEN 20	Polysorbat 20
UTC	Undifferentiated thyroid carcinoma
V	Volt
VEGF	Vascular endothelial growth factor
W	Watt
WT	Wild type
α-SMA	α smooth muscle actin
µg	Microgram
µl	Microliter

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1 Introduction

1.1 Family of relaxin-like peptides

Human relaxin 2 (RLN2) belongs to the family of insulin-like proteins. All members of this family – IGF-I, IGF-II, relaxin 1 (RLN1), relaxin 2 (RLN2), relaxin 3 (RLN3), insulin-like peptide 3 (INSL3), INSL4, INSL5 and INSL6 – demonstrate structural relationships with insulin and, like this protein, are produced as preproforms consisting of signal peptide B-C-A chain. Matured processed forms of relaxin lack the C peptide and the B and A chains are linked by two disulphate bonds (Sherwood et al., 2004).

Initially, relaxin 1 was discovered in pregnant guinea pigs, acting in the uterus and pubic ligaments in preparation for birth and modulating nipple development for lactation postpartum. Human relaxin 2 (RLN2) is specific for higher primates, and like rat relaxin 1 (R1), mouse relaxin 1 (M1), or pig 1 relaxin (P1), is secreted into the blood. Expression of human relaxin 1 (RLN1) was until now detected in few tissues (decidua, trophoblast, and prostate) (Hansell et al., 1991). Exact function of the RLN1 protein is still unknown. It is predicted that the gene of human relaxin 1 arose as a duplication of the human relaxin 2 gene. Homologues of RLN1 were found only in the great apes (Evans et al., 1994). The recently discovered human relaxin 3 (Bathgate et al., 2002) is postulated to be a neuropeptide. Its orthologs were found specifically in the nucleus incertus (NI) of the brains of mice (Bathgate et al., 2002).

Other proteins closely related to RLN1 and RLN2 are insulin-like peptides (INSL) 3, 4, 5 and 6. INSL3 is produced by testicular Leydig cells (Adham et al., 1993) and modulates gubernaculums in the transabdominal phase of testis descent in the fetus (Klonisch et al., 2004). The evidence on involvement of INSL3 in gubernaculums development comes from INSL3 -/- male mice. After birth the animals were found bilateral cryptorchid with testis located near the kidneys. The gubernaculae of INSL3 -/- males were similar to females – having a flat thin bulb with a thin elongated cord (Nef et al., 1999). In other experiment transgenic INSL3 -/- mice, which overexpressed INSL3 in pancreatic beta-cells developed normal transabdominal testis descent by male animals and inguinal hernia and descent of the ovaries to the position near the bladder by females (Adham et al., 2002).

The actions of the three other INSL proteins are unknown. Expression of INSL4 was discovered in the placenta (Koman et al., 1996), INSL5 in the gastrointestinal tract (Conklin et al., 1999) and kidneys (Hsu et al., 1999), and INSL6 in the testis (Lok et al., 2000).

1.1.1 Receptors for relaxin family peptides (RXFP)

As secreted proteins, relaxin-like peptides influence the cells by binding to specific receptors. In 2002, Hsu et al. showed that relaxin 2 binds to and activates two orphan leucine-rich repeats containing the guanine nucleotide binding protein coupled receptors (GPCR) RXFP1 (LGR7) and RXFP2 (LGR8) (Hsu et al., 2002), which are highly conserved across species (Bathgate et al., 2005). Additional tests defined RXFP1 as relaxin 2 and RXFP2 as relaxin 2 and INSL3 receptor (Wilkinson et al., 2005). Expression of RXFP1 shows similar expression pattern across the species. It was found in ovary, uterus, testis, brain, heart, cervix, vagina, nipple and breast (Bathgate et al., 2005, Hsu et al., 2003). There are, however, also differences. In rats and mice, RXFP1 is localised to uterine myometrium while in humans it is mainly expressed in uterine endometrium (Bathgate et al., 2005). RXFP2 was found in testis of rat, mouse and human (Hsu et al., 2003), gubernaculums of rat and mouse, brain of mouse and human and in kidney, thyroid, muscle, peripheral blood cells, bone marrow and uterus of human (Bathgate et al., 2005, Hsu et al., 2003).

The receptor for relaxin 3 has a high sequence identity to somatostatin and angiotensin receptors and therefore is named SALPR (the somatostatin- and angiotensin-like peptide receptor or GPCR135 or RXFP3) but does not bind somatostatin or angiotensin II. RXFP3 is expressed in the brain (Liu et al., 2003b). Ligand binding studies performed with relaxins from many species excluded an interaction of other relaxin family peptides (porcine relaxin, insulin, INSL3, INSL4, INSL6) with this receptor (Liu et al 2003b). The other receptor GPCR142 (named RXFP4) is related to RXFP3 and has similar ligand specifity and may function as INSL5 receptor (Bathgate et al., 2005). RXFP4 is located in the colon, thyroid, salivary gland, prostate, placenta, thymus, testis, kidney and brain (Liu et al., 2003a). All RXFP receptors consist of an N-terminal exodomain, a transmembrane region

with extra- and endocellular loops and a C-terminal endodomain. The extracellular ectodomain of both RXFP1 and its homolog RXFP2 contain 10 leucine-rich repeats and their NH_2-termini domains consist of a conserved low-density lipoprotein receptor-like cysteine-rich motif (low-density lipoprotein class A; LDLa), which is connected with the horseshoe-shaped leucine-rich repeat domains (LRR-domain) and followed by a cysteine-rich hinge region and transmembrane domain (TM) incorporating exo- and intradomain loops. RXFP1 and 2 have longer extracellular domains than RXFP3 and 4, whereas RXFP3 and 4 possess longer C-terminal domains with many potential phosphorylation sites (Bathgate et al., 2005, Wilkinson et al., 2007).

Relaxin 1 and human relaxin 2 both bind RXFP1 and RXFP2, however, they have weaker affinity to RXFP2 (Sudo et al., 2003).

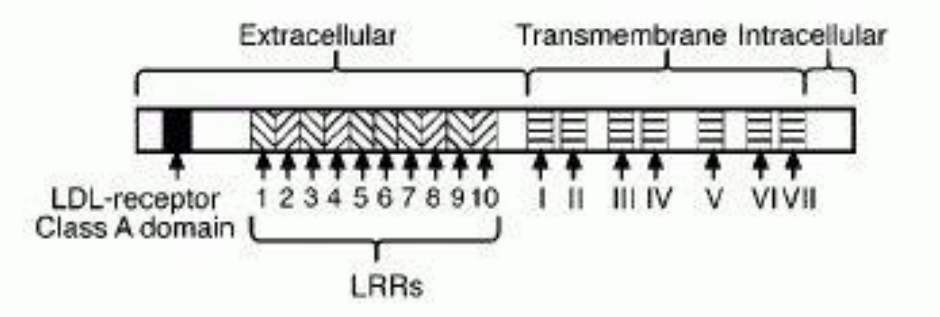


Figure 1: Domain organization of RXFP1 and 2 receptors. The extracellular part consists of the low-density lipoprotein (LDL) receptor class A domain which is followed by leucine-rich repeats (LRRs) 1-10. The transmembrane region and the extra- and intracellular loops is followed by intracellular C-tail (Sherwood et al., 2004)

The binding cassette of relaxin Arg-X-X-X-Arg-X-X-Ile/Val is conserved among many species. Human relaxins 1 and 2, as well as relaxins of porcine and whale, are the most potent relaxins and posses the sequence Arg-Glu-Leu-Val-Arg-X-X-Ile in their binding site. The bioactivity of relaxins derived from rat, shark, dog and horse, which have variations of the human binding cassette sequence, is reduced as compared to human, porcine and whale ones.

Both RXFP1 and RXFP2 bind and respond to RLN1, RLN2 and porcine relaxin. Additionally, RXFP2 respond to INSL3 but not to rat relaxin (Scott et al., 2006). Recent studies display numbers of interaction sites for relaxin and other related peptides within the receptors. Observations performed on membrane-anchored ectodomains indicate the location of the primary binding site in the LRR domain

(Halls et al. 2005b, Yan et al., 2005) and using of chimerical RXFP1/2 receptors (with the RXFP1 ectodomain and RXFP2 TM region) suggested participation of the TM domain (exoloop 2 but not exoloop 1 or 3) in ligand-receptor interactions (Bathgate et al., 2005, Halls et al. 2005b, Sudo et al., 2003). The contact motif for RXFP1 interacts with specific residues of the LRR beta-sheet (Arg B-13 with Glu^{277} and Asp^{279}; Arg B-13 with Glu^{233} and Asp^{231}). Interactions between Ile B-20 and the different residues of beta-sheet additionally stabilise this binding. This initial contact allows the ligand to react with exoloop 2 of the TM domain, which is followed by conformational changes of the ectodomain, coupling of G-protein by the LDLa domain and activation of the adenylate cyclase (Buellesbach et al., 2005, Scott et al., 2006). The investigations of binding sites in RXFP1 receptor performed on chimera also included stimulation with rat relaxin, which binds RXFP1 but not RXFP2. The highest binding affinity after relaxin stimulation was observed for RXFP1. Chimera containing the RXFP1 ectodomain and RXFP2 TM domain (RXFP1/2) showed weaker reaction. In additional studies INSL3, after binding to RXFP1/2 or RXFP2/1 chimeras, initiated weaker increase of cAMP accumulation than it takes place after RXFP2 binding (Halls et al., 2005b). The role of the ectodomain of RXFP1 in ligand-receptor interaction was also studied using the 7BP (binding protein), a soluble domain of relaxin receptor, which interacted with porcine relaxin by composing a strong complex, indicating the presence of binding site included in ectodomain (Hsu et al., 2002). Subcutaneous administration of the soluble ligand binding motif of RXFP1 (LGR7-7BP) in mice abolished the relaxin activity during the late pregnancy as determined by nipple size reduction and delayed parturition (Bathgate et al., 2005, Hsu et al., 2002).

Several isoforms of RXFP receptors arise after alternative splicing in ectodomain. In mice, rats and pigs, the splice variant of RXFP1, which is missing exon 4, has been discovered. This deletion caused a frameshift due to a premature stop codon and consequently truncated secreted protein (RXFP1-truncated). Additionally other splice variants were identified: RXFP2–short, missing the LDLa module and two others secreted RXFP1-short versions – RXFP1-truncate-2 and RXFP1-truncate-3. Characterizations of these splice variants revealed the role of LDLa domain in receptor activation. Furthermore co-transfection of RXFP1 and RXFP1-truncate receptors resulted in the reduction of relaxin-induced signalling, presenting the

truncated protein as antagonistic *in vitro* and suggesting its regulatory function *in vivo* (Scott et al., 2006).

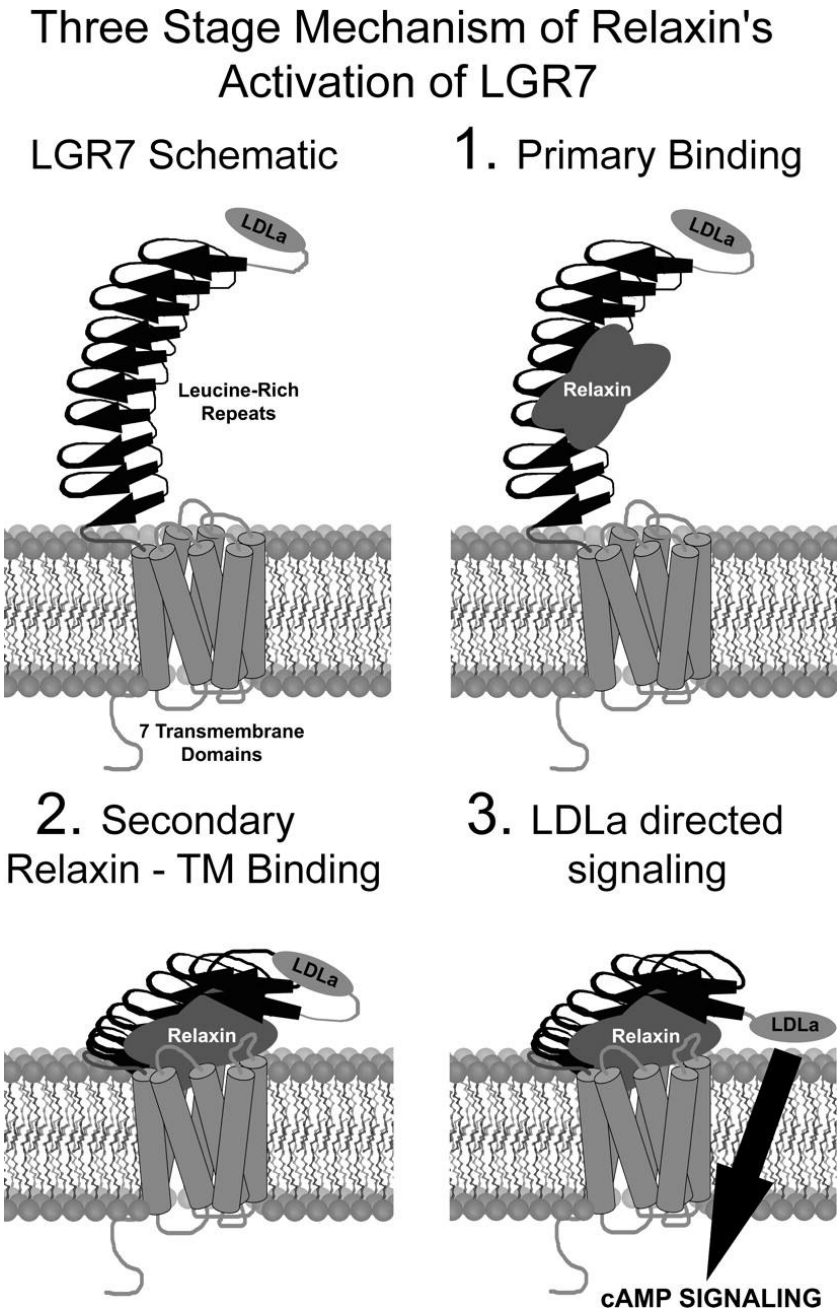


Figure 2: Model of LGR7 activation. Relaxin binds the primary binding site in leucine-rich repeats of the exodomain of the receptor (1), leading to conformational changes, which allow the interaction between ligand and the extracellular loops of transmembrane domain (2). The LDLa module initiates than cAMP accumulation via an unknown mechanism (3) (Scott et al. 2006).

Recognition of RXFP1 and RXFP2 as relaxin and INSL3 receptors provided models to study signalling pathways initiated by these hormones. Accumulation of cAMP in presence of relaxin was noticed in many tissues like mouse pubic symphysis (Braddon et al., 1978), rat uterus (Cheah et al., 1980) as well as in cultures of rat

myometrium (Sanborn et al., 1995) and human endometrium (Fei et al., 1990). More details support studies *in vitro* using HEK293T cell line stably expressing RXFP1 or RXFP2 receptors, which respond with increased accumulation of cAMP upon relaxin stimulation (Bathgate 2005). Further investigations performed by employing a gain of function mutants of both receptors, revealed relaxin/INSL3-independent an increase of cAMP accumulation in these cells. This finding suggests involvement of adenylate cyclase in signalling pathway (Bathgate et al., 2005, Hsu et al., 2000, 2002). Nguyen et al. and Halls et al. supported more evidences for RXFP receptors and their ligands. They demonstrated RXFP1 receptor response as a biphasic cAMP accumulation in THP-1 and HEK293T cells. The complexity of RXFP1 response includes the phosphoinositide 3-kinase (PI3-K) and protein kinase C (PKC) zeta involvement in the second phase of receptor activation (Bathgate et al., 2005, Halls et al., 2005a, Nguyen et al, 2003).

Similar Dessauer et al (Dessauer et al., 2005) suggest the signalling pathway of RXFP-PI3-K-induced cAMP accumulation, also includes PKC actions. This thesis is based on experiments, where inhibition of PKC and relaxin treatment increased the accumulation of cAMP (Dessauer et al., 2005).

Several evidences describe the activation of Erk1/2 as a consequence of RXFP1 activation. Rapid phosphorylation (less than 5 min) of Erk1/2, without changing the total Erk level, was noticed in THP-1, endothelial stromal cells, and primary cultures of human coronary artery and pulmonary artery smooth muscle cells. Moreover inhibition of MEK blocked this effect (Bathgate et al., 2005, Zhang et al., 2002). Much longer time was needed to activate Erk in HeLa cells and human umbilical vein endothelial cells (45–90 min), what suggests a non-direct interaction with RXFP receptors (Dschietzig et al., 2003).

Independly of RXFP1, relaxin also acts on glucocorticoid receptor (GR). In response to endotoxines, human macrophages produce inflammatory cytokines and relaxin reduces this process by interaction with glucocorticoid receptor (GR) (Dschietzig et al., 2004, 2009).

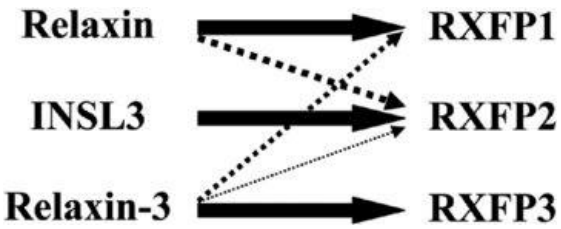


Figure 3: Ligand-receptor interaction inside the relaxin-like peptides (Hartley et al., 2009).

1.1.2 Relaxin in the reproductive tract

The relaxin hormone is regarded as hormone related with pregnancy in several species (Sherwood et al., 2004). In rats, mice, pigs and humans the circulating relaxin hormone is mainly produced in the corpora lutea (Sherwood et al., 1994). In animals, relaxin is accumulated in storage granules of the luteal cells and released into the serum 2–3 days before delivery, whereas in humans it is not accumulated and levels of this hormone peak during the first trimester of pregnancy (Bell et al., 1987; Eddie et al., 1986; Stoelk et al., 1991).

Several animal studies including rats, mice and pigs suggest a role for relaxin in the reduction of frequency of myometrial contractions. Relaxin blocks contractions by enhancing cAMP production in myometrial cells, followed by an increase in PKA activity (Sherwood et al., 1994, 2004). Siebel et al. suggest a role for relaxin in preventing spontaneous and oxytocin-stimulated uterine contractions at the level of signalling pathways (Siebel et al., 2003). In humans, relaxin is not expressed in the myometrium; however, it was discovered in endometrium, where its production may play a significant role in early pregnancy (Bond et al., 2004).

Relaxin plays a crucial role during delivery. Experiments with rats and pigs showed the influence of relaxin on the duration of delivery. It is known that 25% of relaxin-KO mice could not deliver their pups normally. In one (12.5%), all pups either died in the uterus or were stillborn. RXFP1-KO mice delivered 162 pups, and in the morning of birth 25 (~15%) were found dead. In rat, mouse and pig pregnancies, relaxin plays a key role in the growth of the cervix (Sherwood et al., 2004) by increasing proliferation (Lee et al., 2003) and inhibiting apoptosis (Zhao et al., 2001) of stromal cells (Sherwood et al., 2004).

Experiments on rats underlined the role of relaxin in nipple development by influencing collagen and elastin organisation (Hwang et al., 1991, Sherwood et al., 1994).

1.1.3 Relaxin in non-reproductive tissues

Another of the multiple effects of relaxin is reorganisation of extracellular matrix (ECM). Investigations performed *in vitro* on dermal fibroblasts and in lung fibroblasts revealed relaxin-dependent reduction of collagen synthesis and from the other hand induction of collagenases production (Cooney et al., 2009, Unemori et al., 1990; 1993). In fibroblasts obtained from neonate rats, the relaxin alone did not change the collagen expression, observed after TGF-β or Ang II stimulation only. However, the changes in collagen deposition were reduced by addition of relaxin to TGF-β or Ang II treatments. This coincided with increased expression of MMP-2 and reduced content of collagen (Samuel et al., 2004). In kidney of bromoethylamine (BEA) -treated rats, relaxin-mediated decrease in collagen deposition, typical for BEA treatment, led to reduction of fibrosis extent. However, as revealed by zymographic analysis, the reduction of collagen deposition was not an effect of increased MMP-2 activity (Garber et al., 2001). In hepatic stellate cells (HSC) relaxin diminished the collagen deposition in dose-dependent manner. This reduction was not an effect of decrease in collagen expression but lower levels of physiological inhibitor of metalloproteinases - TIMP-1. Relaxin-mediated reduction in collagen deposition was also observed *in vivo*, in rat model with hepatic fibrosis (Williams et al., 2001).
Expression of relaxin receptor RXFP1 was found in the rat (Hsu et al., 2000, Kompa et al., 2002), mouse (Mazella et al., 2004) and human (Hsu et al., 2002) heart, confirming the heart as a target tissue for relaxin. Relaxin itself (relaxin 1 and relaxin 2) was detected in hearts of human and mouse but only on transcriptional level (Bathgate et al., 2002, Dschietzig et al., 2001). Both relaxins were also detected in mammary arteries and saphenous veins, and in human atrial and ventricular tissues (Dschietzig et al., 2001). In patients with congestive heart failure (CHF), elevated levels of relaxin were proposed as a marker for assessing the severity of CHF (Samuel et al., 2003).

In a variety of species relaxin induced positive chronotropic effect both *in vivo* and *in vitro* (Coulson et al., 1996, Kakouris et al., 1992, Parry et al., 1990, 1998, Samuel et al., 2003, Tan et al., 1998) and some studies also demonstrated the inotropic effect of relaxin on mammalian hearts (Kakouris et al., 1992, Kompa et al., 2002, Piedras-Renteria et al., 1997, Samuel et al., 2003, Tan et al., 1998). It was shown that relaxin increased the rate of spontaneous contractions in hearts (Bani Sacchi et al., 1995), or in the isolated right atria (Kakouris et al., 1992, Tan et al., 1998, Ward et al., 1992), where already nanomolar concentrations of human relaxin were able to provoque the chronotropic and inotropic effects, displaying the human relaxin as more potent than relaxin of rat in the heart (Kakouris et al., 1992, Tan et al., 1998). On the other side in rats with myocardial infarction (MI) in cardiatic failure, relaxin showed only the inotropic effects (Kompa et al., 2002).

Examinations of relaxin activity in other parts of cardiovascular system demonstrated its role in coronary blood vessels as a potent vasodilator (Bani et al., 1998, Bani Sacchi et al., 1995, Fisher et al., 2002). However, this activity depends on endothelium and is absent in endothelium-intact aortic rings precontracted with noradrenaline (Reid et al., 2001). Moreover, in the rat model of ischemia-reperfusion-(IR)-induced myocardial injury, relaxin reduced degranulation of mast cells, which in IR release mediators (histamine, serotonin and leukotrienes) that are suggested to participate in damaging the tissue (Masini et al., 1997). Inflammation processes such as IR led to tissue injury and the migration of neutrophils, which than released reactive oxygen and lysosomal enzymes (Nistri et al., 2003). At the same time, inflammatory mediators stimulated the expression of several adhesion molecules in endothelial cells (Bani et al., 1995).

Strong evidence on the role of endogenous relaxin comes from relaxin lacking KO mice (Zhao et al., 2001). In male RLN -/- mice the atrial hypertrophy and impeeded left ventricular diastolic filling and venous return were observed (Du et al., 2003). The same mice display increased gene expression of pro-collagen (type I), collagen content and concentration (Du et al., 2003), which caused lower ventricular chamber elasticity. Female mice did not display any detectable changes in cardiatic phenotype.

1.1.4 Relaxin in cancer

Relaxin plays also significant role in cancer. In the mammary gland, relaxin 1 and relaxin 2 are associated with both physiological and neoplastic events (Bryant-Greenwood et al., 1994, Mazoujian et al., 1990, Silvertown et al., 2003b, Tashima et al., 1994,). RLN2 was detected in all neoplastic breast tissues, but only in few non-neoplastic, when RLN1 was expressed in 75% of neoplastic tissues but only in 12.5% of normal tissues (Tashima et al., 1994).

Relaxin influences also the growth and the differentiation of tumour cells. Experiments *in vitro* demonstrated biphasic effect of relaxin on MCF-7 breast cancer cells (Bigazzi et al., 1992). Under experimental conditions relaxin increased cells proliferation in concentrations of $2x10^{-10}$ to $8x10^{-10}$ M. Higher concentrations of relaxin led to a dropping of proliferation but also to differentiation of the cells. Strong evidence indicated that at concentrations of 10^{-9} and 10^{-6} M relaxin influenced differentiation of MCF-7 cells cocultured with human myoepithelial cells (Bani et al., 1994). The same cell line (MCF-7) was transplanted in nude mice, which were than treated with porcine relaxin (10 µg/day) for 19 days. Also this experiment displayed relaxin-dependent induction of cell differentiation forwards myoepithelial-like and epithelial-like cells. Differentiation of the cells was advanced, showing the changes in organelles, cytoskeleton and intracellular junctions (Bani et al., 1999, Silvertown et al., 2003b).

Further studies demonstrated that relaxin treatment induces the activity of matrix metalloproteinases in breast cancer cell lines MCF-7 and SK-BR3, and led to enhanced invasiveness of the cells (Unemori et al., 1990). Additional EGF stimulation augmented the effect of relaxin in MCF-7 cells (Unemori et al., 1990). With regard to the response of canine mammary carcinoma cells CF-33 to relaxin stimulation with increased laminin migration, no effect on proliferation was noticed (Silvertown et al., 2003b).

Clinical investigations supported further evidence on a possible role of relaxin in cancer development. Examinations performed by Binder et al. (Binder et al., 2004) revealed increased levels of relaxin in sera of breast cancer patients with metastasis.

1.2 Cytoskeleton and invasion of tumour cells

1.2.1 Plasticity of migrating cells

The morphology and plasticity of eukaryotic cells depend on their cytoskeleton organisation. This consists of three types of filaments:

- intermediate filaments, responsible for the mechanical stress and strength of the cells;
- microtubules, which influence the membrane-enclosed organelles position and intracellular transport; and
- actin filaments, determining the shape of cells and their migration potential.

All filaments are polypeptides of smaller protein subunits, which can diffuse quickly within a cytoplasm, migrate to the other end of the cell and assemble to the filament. Microtubules consist of tubulins. Tubulins are dimers of alpha- and beta-tubulins bounded with each other and each of them can bind one GTP molecule. Consisting of 13 parallel alpha- and beta-tubulins, protofilament microtubules create stiff, cylindrical structures. Most of the actin proteins in eukaryotic cells constitute the monomers (globular – G-actin; 42 kDa) (Alberts et al., 2002, Ayscough et al., 1998, Dos Remedios et al., 2003), which may bind as subunits of the polypeptide chain (Alberts et al., 2002). However, only a small number of actin subunits exist in the polymerisated form, creating a filament network (filament – F-actin) (Ayscough et al., 1998, Dos Remedios et al., 2003). In the cell, actin filaments may exist as two parallel protofilaments twisted around each other in a right-handed helix (Alberts et al., 2002). Moreover, several filaments are cross-linked and bundled together, creating very strong large-scale structures.

The subunits of all filaments are joined in protofilaments. Multiple protofilaments build the polymers. Protofilaments such as actin or intermediate filaments usually twist around one another in a helical fashion. This structure gives them greater resistance to mechanical stress (Alberts et al., 2002, Nogales et al., 2006).

The usefulness of cytoskeletal filaments depends on the accessory proteins which link the filaments to each other or to other cell components. The accessory proteins include motor proteins that either move organelles along the filaments or the filaments themselves. The accessory proteins bind to the filaments or their subunits to determine the sites of assembly of new filaments, regulate the partitioning of

polymer proteins between filament and subunit forms. By controlling these processes, the accessory proteins bring the cytoskeletal structures under the control of extracellular and intracellular signals, and by this enable the eukaryotic cells to move (Alberts et al., 2002).

Cytoskeletal integrity is also sensitive to toxins produced by plants, fungi or sponges in self-defence. The toxins bind free subunits or the filaments making the assembly-reassembly processes impossible. For example, *latrunculin* binds to and stabilises the actin monomers, causing the depolymerisation of filament. *Phalloidin* binds to and stabilises the filament, making depolymerisation impossible. The toxins are often used in biological studies of various processes such as cell movement (Alberts et al., 2002).

1.2.2 Dynamics of actin cytoskeleton

Locomotion of the cells depends on the speed of actin reorganisation in the front of the cell, where monomers polymerise and form actin filaments (Wang et al., 1985) and push the leading front forward (Mitchison et al., 1996, Pollard et al., 2003).

Dynamic of the filaments requires both the ability to form noncovalent polymers and catalyse the hydrolysis of ATP. ATP is joined to free subunits, where its hydrolysis is very slow. However, when the subunits are incorporated into filament, hydrolysis proceeds quickly. Soon after integration, the free phosphate group (P_i) is released and nucleoside diphosphate (ADP) remains trapped in the filament. Together with releasing the P_i group, much of the free energy of the phosphate-phosphate bound cleavage is stored in the polymer (Fig. 4) (Alberts et al., 2002, Pollard et al., 1986).

Actin filaments are polarised structures consisting of minus (pointed) and plus (barbed) ends. Growth and elongation of filaments can take place on both sides, however, at the plus end is about 10 times faster (Lorenz et al., 2004). Both ends can also depolymerise. Such a situation takes place when the concentration of free subunits in the cytoplasma drops. In such circumstances, the plus end also depolymerises faster than the minus end (Alberts et al., 2002).

When the concentration of the free subunits is intermediate (higher for ATP and lower for ADP forms), the subunits are added at the plus (ATP) end and lost at the minus (ADP) end. This process is called filament *treadmilling* (Small et al., 1995).

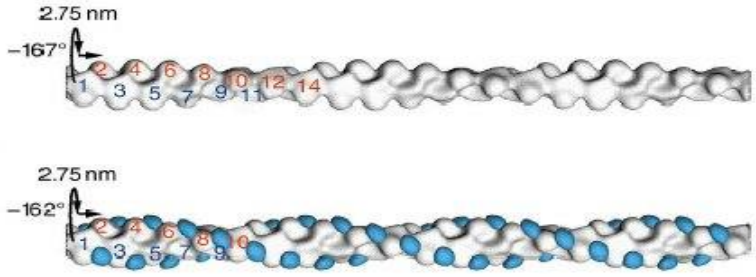


Figure 4: Binding the cofilin (blue) changes the rotation of actin filament helix and destabilises the actin-actin binding (Bamburg et al., 1999, Ono et al 2003).

One of the proteins responsible for defragmentation is cofilin. At the pointed end, cofilin binds the actin filaments between two molecules, destabilising the filament and in consequence deassembling it (Carlier et al., 1997, Galkin et al., 2003, Nishida et al., 1984, Renoult et al., 1999). By binding to actin filaments, cofilin forces the filament to twist more. Such mechanical stress weakens the bindings between actin subunits and makes the filament more unstable. The result of cofilin activity is an increased rate of *treadmilling*. Cofilin is then thought to be the *treadmilling* factor.

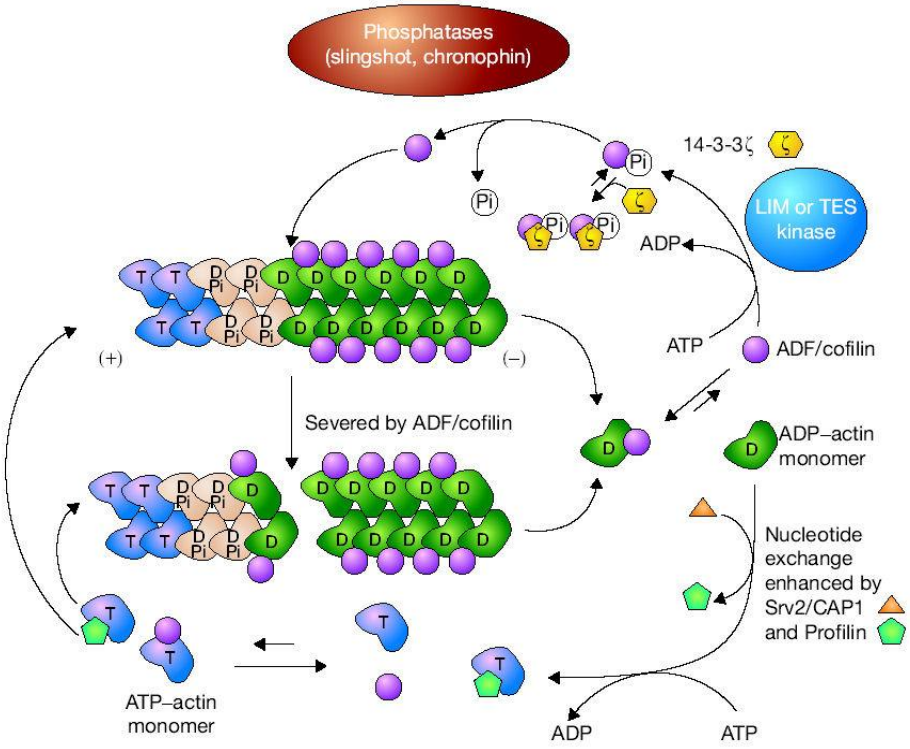


Figure 5: Actin treadmilling. ADF/cofilin destabilizes actin by binding the pointed end of the filament what at the end leads to defragmentation of actin. After exchanging ADP to ATP actin monomers are joined to the barbed end (Wiggan et al., 2005).

Since cofilin preferentially binds ADP-containing filaments over ATP-bounded filaments (Carlier et al., 1997), and the hydrolysis of ATP in filaments is slower than the assembly of new subunits, the newly formed ATP-rich filaments are resistant to cofilin depolymerisation. By binding the older ADP-rich filaments, cofilin dismantles the older filaments, ensuring a rapid turnover of actin filaments (Alberts et al., 2002).
Both proteins (actin subunit and cofilin) are together until the exchange of ADP into the ATP in actin monomers (Nishida et al., 1985), which are now ready for polymerisation on the barbed end.

1.2.3 Cofilin regulating factors

The activity and function of cofilin depend on many factors, such as pH or phosphorylation. It is known that in acidic pH (< 6.8) cofilin stabilises actin, whereas in higher alkaline environments (pH > 7.3) it depolymerises actin filaments. The physiological meaning of this state is still unknown (Bamburg et al., 1999).
The phosphorylation status, where phosphorylated cofilin is unable to bind the actin (Morgan et al., 1993), plays an important role in cofilin activity. The known cofilin kinases are TESK – for testis (Røsok et al., 1999, Toshima et al., 1995) – and the ubiquitously expressed LIMK1/2 (Foletta et al., 2004, Mizuno et al., 1994). The LIMKs are controlled by the Rho-GTPases family (Rac, Rho, Cdc42) (Amano et al., 2001, Yang et al., 1998). The Rho acts on the LIMK1 (Ohashi et al., 2000) and LIMK2 (Amano et al., 2001) by ROCK phosphorylation, when the Rac and Cdc42 can control the activity of LIMK through PAK1 (Edwards et al., 1999) or PAK4 (Amano et al., 2001). The lesser-known effector of LIMK is Ras protein, which probably has an influence on the dephosphorylation of LIMKs (Nebl et al., 2004).
The dephosphorylation of cofilin is rarely studied. Takuma et al. (Takuma et al., 1996) revealed the dephosphorylation of cofilin through conventional serine/threonine phosphatases types 1, 2A and 2C (PP1, PP2A, PP2C) and Ambach et al. (Ambach et al., 2000) described the first evidence *in vivo* correlating PP1 or PP2A with cofilin dephosphorylation. The other specific cofilin phosphatase is Slingshot (SSH) (Niwa et al., 2002). However, regulation of SSH through the Rho-GTPases family and regulation of cofilin phosphorylation is still under study.

1.3 Protease-related migration

One important factor for the migratory behaviour of the cells is their interaction with ECM components. ECM consists of protein fibres sunk in a hydrated gel of a glycosaminoglycan (GAG) chains network. GAGs are polysaccharides covalently bound to proteins, to create proteoglycan molecules (Alberts et al., 2002).
Proteins of ECM are formed in fibres. Such configuration gives them the strength and form of the matrix, and the surface for cells to adhere. One of such protein is elastin, which creates the fibre network as well as the sheets providing the elasticity to the matrix. Fibronectin assembles into fibres only in the assistance of other proteins such as integrins where it creates fibrillar adhesion sites on the surface of the cells. Fibrillar collagens (types I, II, III, V, and XI) create long fibrils, highly organised in ECM. The collagen fibrils interact with one another via the fibrils-associated collagens (types IX and XII), which are connected to the surface of the fibres.
ECM components are degraded by proteolytic enzymes, which are usually metalloproteinases. Activity of one type of those proteases (collagenases) leads to the creation of another protein - gelatin. Collagenases cut fibrillar collagen in specific sites, generating smaller fragments which denaturate to gelatin at body temperature (Alberts et al., 2002).
Invasive cells produce several proteases, which digest the ECM proteins surrounding them, opening the way for cells to invade.

1.3.1 Metalloproteinases and their inhibitors

Alteration of extracellular architecture requires the coordinated interaction of various factors, such as proteolytic or adhesion molecules (Nelson et al., 2000). Matrix metalloproteinases (MMPs), a disintegrins and metalloproteinases (ADAMs) and Catthepsins are proteases capable of degrading different substrates of the extracellular matrix.
In physiological conditions, activity of MMPs was noticed in development and in differentiation of tissues (Ghajar et al., 2008, Krane et al., 2008). Pathological studies revealed their expression in certain diseases such as rheumatoid arthritis (Murphy et al., 2008). Over the past few years, their outstanding role in tumourigenesis was also discovered. Due to their ability to degrade ECM

components, the proteases play an important role not only in tumour development but also in the invasion and metastasis of tumour cells (Stöcker et al., 1995).

At the transcriptional level, expression of most MMPs (MMP-1, -3, -7, -9, -10, -12, -13 and -19) is induced by several stimuli, such as growth factors, cytokines, oncogenes, hormones and ECM proteins (Johansson et al., 2000). By extracellular signal, the MMPs activate the AP-1 transcription factor complexes, which then bind to the promoter of the MMPs and stimulate transcription (Johansson et al., 2000).

On the protein level, MMPs, synthesised as zymogens, require the activation of proenzymes. *In vitro* this process, known as the cysteine-switch model, can be regulated by high temperature, low pH, denaturating agents and others, and is based on the disturbance of the zinc ion, and cysteine-sulphydryl group (Folgueras et al., 2004, Morgunova et al., 1999, Nagase et al, 1997, van Wart et al., 1990). The *in vivo* propeptide domain is removed by another proteolytic enzyme. Knowledge of this pericellular mechanism of activation is supported by MT1-MMP studies, displaying this enzyme as an activator of some proMMPs including proMMP-2 (Morrison et al., 2001, Nie et al., 2003, Strongin et al., 1995, Tokuraku et al., 1995, Zucker et al., 2003).

Metalloproteinases are inhibited by general inhibitors localised in plasma and tissue fluids (alpha2-macroglobulin) or by more specific tissue inhibitors of metalloproteinases (TIMPs). Three of four vertebrate TIMPs exist as secreted proteins (TIMP1, -2 and -4) and one is anchored to the ECM (TIMP3). All TIMPs can inhibit active MMPs, however, TIMP1 weakly inhibits MMP-19 and MT1-MMPs (Lee et al., 2003), and TIMP3 preferentially blocks certain ADAMs and ADAMTSs (a disintegrin and metalloproteinases with trombospondin motive) (Amour et al., 2000, Kashiwagi et al., 2001). However, most important in living organisms is maintaining the balance between metalloproteinases and their inhibitors. Previous reports demonstrated that affecting one of these factors results in serious physiological consequences. For example, the over-production of TIMPs is involved in the reduction of metastasis (Declerck et al., 1994) and vice versa, their decreased expression correlates with growth and tumour progression (Khokha et al., 1989).

1.3.2 Matrix metalloproteinases

Bioinformatical investigations revealed the ability of MMPs to cleave any component of ECM and basement membrane, allowing tumour cells to invade the stromal matrix (Brinckerhoff et al., 2002, Folgueras et al., 2004). Genomic studies revealed 24 distinct genes encoding the proteins of the MMP family (Folgueras et al., 2004, Puente et al., 2003). According to the substrate MMPs are divided to the human collagenases, gelatinases, stromelysins, matrilysins and membrane type MT-MMPs (Folgueras et al. 2004, Johansson et al., 2000).

Collagenases (MMP-1, MMP-8 and MMP-13) are capable of degrading native fibrillar collagens types I, II, III, V and XI in ECM. MMP-1 preferentially degrades type III collagen, MMP-8 degrades type I and MMP-13 prefers type II.

Additionally, MMP-13 also digests collagens types IV, IX, X and XIV, fibronectin, laminin, agrecan core protein, fibrillin-1 and serine proteinase inhibitors. Moreover, all collagenases also display gelatinolytic activities, which are the strongest in MMP-13 (Johansson et al., 2000).

Both stromelysins (MMP-3 and MMP-10) are expressed by fibroblasts and by normal and transformed epithelial cells *in vivo* and *in vitro*. MMP-3 and MMP-10 degrade broad spectrum of ECM components, such as collagens type IV, V, IX and X, proteoglycans, gelatin and fibronectin. Interestingly, MMP-11 (stromelysin-3) does not degrade any ECM components, but digests several inhibitors.

Next to the gelatin, Gelatinase A (MMP-2) and Gelatinase B (MMP-9) degrade collagens type IV, V, VII, X, XI and XIV, elastin and the proteoglycan core protein. Moreover, MMP-2 can degrade native collagen I and MMP-9 in acidic environment cleaves N-terminal telopeptide of collagen type I (Johansson et al., 2000).

The membrane type metalloproteinase-1 (MT1-MMP) activates MMP-2 by interaction with the MMP-2/TIMP2 complex. Substrates for this protein are collagens type I, II, and III, gelatin, fibronectin, laminin-1, vitronectin, cartilage proteoglycans and fibrillin-1. The second member of this subfamily MT2-MMP is the next activator of proMMP-2 and proMMP-13. Its ECM substrates are laminin, fibronectin and tenascin. MT3-MMP also activates proMMP-2. It hydrolyses gelatin, casein, collagen III and fibronectin. MT5-MMP activates the latent MMP-2 and its shedding from the cell membrane suggests its function as a membrane-bound and soluble proteinase

(Johansson et al., 2000). In addition to the ECM proteolytic activity, metalloproteinases can release or process other molecules (Vu et al., 2000).

1.4 Physiological function of MMPs and ADAMs

MMPs are implicated in many physiological and pathological processes which require the disruption of ECM, or release and process bioactive molecules, which widen their importance in biological events (Vu et al., 2000). Studies of embryonic growth and tissue morphogenesis revealed the involvement of MMPs in collagenolytic activity for major developmental events, such as tail restoration during metamorphosis in tadpoles (Brinckerhoff et al., 2002, Gross et al., 1962) or invasion of trophoblasts in the early implantation stages (Alexander et al., 1996), skeletal and connective tissue development as well as in angiogenesis (Holmbeck et al., 1999, Zhou et al., 2000) or in the wound healing process (Bullard et al., 1999, Pilcher et al., 1997). Many studies revealed the significant function of MMPs in angiogenesis (Brooks et al., 1994, Folgueras et al. 2004) and vascularisation (Folgueras et al. 2004, Itoh et al., 1998, Lambert et al., 2003).
Latest investigations also describe the role of ADAMs in several physiological processes. Recent studies demonstrated their potential role in adhesion and cell-cell interaction (Rawlings et al., 1995), inhibition of angiogenesis (Iruela-Arispe et al., 1999) as well as their involvement in inflammatory processes (Miles et al., 2000) or kidney, uterus and ovaries development (Miles et al., 2000).

1.4.1 MMPs and ADAMs in cancer

In cancer biology, MMPs are involved in tumour growth in early stages by processing molecules, influencing the microenvironment formation and, in invasive stages, by disrupting ECM. In the first phase MMPs activate growth factors (Egeblad et al., 2002, Hojilla et al., 2003) joined to proteins, such as insulin-like growth factors (Mañes et al., 1997), or anchored to the cell membrane as proproteins (Yu et al., 2000), repress apoptosis of tumour cells, antagonize chemokines

produced by host immune response (Li et al., 2002, McQuibban et al., 2000, van den Steen et al., 2002) or release angiogenic factors (Egeblad et al., 2002).

Many examples show the upregulation of MMPs in tumour tissues. Higher expression of MT1-MMP and activation of MMP-2 were observed in several cancers such as the lung (Nawrocki et al., 1997, Polette et al., 1996, Sternlicht et al., 1999, Tokuraku et al., 1995), pancreas (Imamura et al., 1998), gastric (Bando et al., 1998, Mori et al., 1997, Nomura et al., 1995, Ohtani et al., 1996), thyroid (Nakamura et al., 1999), bladder (Kanayama et al., 1998), head and neck (Yoshizaki et al., 1997), brain (Forsyth et al., 1999, Nakada et al., 1999, Yamamoto et al., 1996), ovaries (Afzal et al., 1998, Fishman et al., 1996) and cervix (Gilles et al., 1996). MT1-MMP expression in glioma cells correlates with tumour status (Fillmore et al., 2001). MMP-13, MMP-7 and MT1-MMP in normal keratinocytes are markers of processing the transformation into malignant cells, and MMP-2 is a marker of malignant transformation of cervical epithelial cells (Johansson et al., 2000).

During the invasion process, however, tumour cells need to cooperate with stromal cells and inflammatory cells. In SCCs (squamous cell carcinoma), matrilysin (MMP-7) is expressed only by tumour cells, and MMP-13 mainly by tumour cells. On the other hand, MMP-2 is secreted only by stromal fibroblasts and MMP-1 mainly by stromal fibroblasts. MT1-MMP is expressed by both tumour and stromal cells and MMP-9 by tumour and inflammatory cells. Colocalisation of cells expressing MT1-MMP and MMP-2 with tumour cells producing MMP-13 consequently creates optimal conditions for activation of MMP-13 (tumour) and MMP-2 (stroma) and invasion. Expression of other proteases such as MMP-3 or MMP-7 augments the invasion probability in tumour-driven proteolytic cascades, in which MT1-MMP or MMP-3 can activate MMP-13 (Johansson et al., 1999, 2000, Yoshizaki et al., 1997). Analysis of SCC cells from different organs underlines the role of MMPs in aggressivity of tumour cells. MMP-1 is correlated with poor prognosis in colorectal and oesophageal cancer, and MMP-2 and MMP-3 is related to lymph node metastasis and vascular invasion in the SCC of the oesophagus (Johansson et al., 2000). Abundant expression of MMP-13 in the SCC of the head, neck and vulva is connected with their metastatic potential (Johansson et al., 1997, 1999). MMP-2 in cervical SCC cells is associated with a poor prognosis (Davidson et al., 1999) and MMP-11 correlates with the increased local invasiveness in head and neck SCCs (Johansson et al., 2000).

Direct effect of MMPs on tumour growth was shown using 3D collagen-matrix gels, where tumour cell expansion was induced by MT1-MMP overexpression without changes in soluble MMP production (Hotary et al., 2003). These data could not be repeated in 2D systems, underlining the importance of surrounding ECM on cell behaviour (Cukierman et al., 2001). MMPs also play a role in tumour angiogenesis by induction or activation of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) or transforming growth factor-beta (TGF-beta) (Belotti et al., 2003, Bergers et al., 2000, Mohan et al., 2000, Sounni et al., 2002). Other evidence of the role of MMPs in tumour vascularisation is displayed in the induction of MMP-9 in tumour macrophages and endothelial cells and in the promotion of lung metastasis (Yu et al., 2000) or increasing neovascularisation by MMP-9 derived from the host cells in ovarian carcinomas (Huang et al., 2002).

Analysing the influence of MMPs on cell apoptosis *in vivo* showed that mice deficient in MMP-2, MMP-3 or MMP-9 displayed lower levels of apoptosis induced by TNF-alpha (Wielockx et al., 2001). Mice lacking MMP-7 showed decreased tumourigenesis. MMP-11 KO mice, after chemical mutagenesis, demonstrated impaired tumour formation, and knocking out the MMP-2 resulted in reduced tumour growth and a weaker metastatic ability in Lewis lung carcinomas and B16-BL6 melanoma cells (Johansson et al., 2000). Recently, MMP-9 was also correlated with the suppression of angiogenesis and tumour growth in mice (Hamano et al., 2003).

Many reports also show an overexpression of ADAMs in tumour tissues. Increased expression of ADAM8 was noticed in most tissues and serum samples from lung carcinoma patients. Moreover, tumour cells transfected with ADAM8 showed increased invasive potential. Human renal cell carcinomas and human primary brain tumours (such as astrocytomas) expressed increased levels of ADAM8. Additionally, its expression in brain tumours was correlated with invasiveness (Rawlings et al., 1995). Similarly, ADAM9 is overexpressed in several cancers such as pancreatic cancer, stomach cancer, skin melanoma, hepatocellular carcinoma and in breast cancer. In non-small lung carcinoma cells ADAM9 enhances cell adhesion and invasion. Secretion of ADAM9 by stromal cells induces the invasion of colon carcinoma cells *in vitro* (Rawlings et al., 1995). ADAM12 is upregulated in stomach, liver and colon cancers. An active ADAM12 is expressed in glioblastomas, and its levels in urine correlate with breast cancer progression. Its expression by tumour

cells inducts apoptosis of stromal cells. The role of ADAM15 in tumours is unclear. Its upregulation was shown in many cancers (breast, prostate, stomach and lung). However, in ovarian cancer it decreases adhesion and motility of cells (Beck et al. 2005). Recent studies demonstrated that ADAM23 plays a role in adhesion and cell-cell interaction in normal and pathological processes, including the progression of neural origin tumours (Rawlings et al., 1995).

ADAMTS-1 is highly upregulated in breast cancers with increased metastatic potential. Full-length ADAMTS-1 proteins promote metastasis of murine mammary carcinoma and Lewis lung carcinoma cells, but its fragments inhibit metastasis rather than promote. ADAMTS-4 and ADAMTS-5, both aggrecanases, are overexpressed in proliferating glioblastoma cells, probably contributing to their invasive potential (Mochizuki et al., 2007).

Additionally, expression of ADAMTS-5 is correlated with the malignancy grade of chondrosarcoma cells (Sugita et al., 2004).

The activity of metalloproteinases depends on the expression of their inhibitors. TIMP2 inhibits the invasion of HT-1080 fibrosarcoma cells *in vitro* and TIMP1 reduces metastasis in gastric cancer and the invasion of astrocytomas or mammary carcinoma cells (Johansson et al., 2000). TIMP1 also suppresses tumour formation in the liver when crossed with transgenic mice expressing the SV40 T antigen, which develop hepatocellular carcinomas. The protective activity of TIMP1 was also noticed in brain tumour formation (Johansson et al., 2000). Migrating cells that expressed TIMP1 couldn breach vessel walls and leave the bloodstream but after reaching the secondary place, they could not create metastasis (Brinckerhoff et al., 2002). There are controversial data on TIMP2 activity in two different melanoma cell lines. TIMP4 on the other hand inhibits the invasion of breast carcinoma cells *in vitro* and tumour growth *in vivo*. TIMP3 overexpression reduces the tumour growth of stable transfected colon carcinoma cells.

There are many reports underlying the role of metalloproteinases in thyroid cancer development. Follicular and papillary thyroid carcinoma cell lines (follicular thyroid carcinomas FTC-133, FTC-236, FTC-238 and papillary carcinoma TPC-1) express and secret proform of MMP-2. Increased maturation and activity of this protease was detected in all these carcinoma cell lines, except TPC-1, after EGF stimulation, and was correlated with the increased expression of MT1-MMP. Expression of the other

gelatinase MMP-9 was very weak and its activity was detected in only two cell lines (FTC-238 and TPC-1) (Yeh et al., 2006).

Analyses of serum from patients with various types of thyroid carcinomas revealed a relationship between the levels of MMP-2 in serum and malignancy of tumours (Pasieka et al., 2004). Examinations of thyroid tissues displayed increased expression of both gelatinases (MMP-9 and MMP-2) and their inhibitors TIMP1 and TIMP2 in follicular and papillary thyroid tumours, whereas it was not found or was very weak in benign tissues (Spens, 2001). Other studies showed the presence of MMP-2 in widely invasive follicular carcinoma (83%) and minimally invasive carcinoma (80%). Expression of MMP-2 in follicular adenomas and adenomatous goiter was negative (5.3% and 0% respectively). MT1-MMP and TIMP2 were expressed in almost all tissues. The other gelatinase (MMP-9) and the collagenase (MMP-1) were also detected in samples of all tested carcinoma tissues. MMP-7 displayed expression patterns similar to MMP-2 (Cho Mar et al., 2006).

1.4.2 MMPs and relaxin

Expression and activity of MMPs is also regulated by hormones of relaxin-like family, and is cell-type dependent. While in cervical fibroblasts MMPs' activity was induced by relaxin, in endometrial cells proMMP-1 protein was noticed to be reduced (Palejwala 2001, 2002, Silvertown et al., 2003b). In breast cancer cell lines MCF-7 and SK-BR3 relaxin induced expression of MMP-2, MMP-9 and MMP-14 and increased cellular migration (Binder et al., 2002, Silvertown et al., 2003b). Increased invasive behaviour as an effect of relaxin was noticed also in canine mammary cell line CF33.Mt (Silvertown et al., 2003a, 2003b). On the other hand relaxin (100 ng/mL) decreased migration of human mammary cancer cell line MDA-MB-435 after 48 hr (Silvertown et al., 2003b). Exposition of primary human cervix fibroblasts to porcine P1 relaxin enhanced secretion of several metalloproteinases (Hwang et al., 1996), however, treating immature pigs with relaxin led to a decrease of MMP-2 production (Lenhart et al., 2001) and an increase of its inhibitors TIMPs1 and 2 (Sherwood et al., 1994).

A positive influence of relaxin signalling on gelatinases was also noticed in other cells. In placental extravillous trophoblasts activity of MMP-2 and MMP-9, but not

MMP-3 (stromelysin), was increased (Maruo et al., 2007) and in the fibroblasts of periodontal ligament production of MMP-2 was relaxin concentration-dependent and activity of this protease was unaffected (Henneman et al., 2008). Relaxin also induces tissue remodelling in THP-1 cells by increasing the expression and activity of MMP-9 but not MMP-2 (Ho et al., 2007).

1.5 Cathepsins family

Cathepsins are family of lysosomal proteases composed of heavy and light chains connected with each other via disulphate bounds (Turk et al., 2001). Although most of them are defined as endopeptidases (Cathepsins L, V, S, K, F, B, H), proteins with exopeptidases or both activity (B, H, C) are also found in this family (Turk et al., 2001).

1.5.1 Control of cathepsins activity

Like most proteases, cathepsins are synthesised as zymogens. Their activation requires removing the N-terminal propeptide and can be facilitated by other peptidases (except pepsin), Cathepsin D or is achieved autocatalytically at acidic pH (Turk et al., 2000). Cathepsin S and L are synthesised in neutral pH as proenzymes. Lowering the pH leads to proteolysis and separation of the profragment and the matured, active protein (Lennon-Dumenil et al., 2002, Villadangos et al., 2000).
The activity of cathepsins is controlled by cystatins (Turk et al., 1991, 2001, Turk et al., 1997, 2000) or p41, a selective inhibitor of Cathepsin L (Bevec et al., 1996, Guncar et al., 1999, Turk et al., 2001). To keep activity in neutral pH, matured cathepsins bind p41 (Fiebiger et al., 2002). In pH 5.5 and pH 7.2, Cathepsin L is inhibited by binding testican-1. In neutral pH, this protein stabilises Cathepsin L and slows down pH-induced denaturation so the protease remains active longer (Bocock et al., 2003). In the pancreatic cell line HPC-YP, the secreted form of the enzyme was stable at pH 7.4 and larger than in other tissues, (about 68 kDa). This could be a result of the complex between the pro- and matured form of Cathepsin L. Compared with liver Cathepsin L, the pancreatic enzyme reacted differently with several inhibitors. Composition of complexes can be the way to

prolong the digestive potential of the enzyme in cancer cells (Yamaguchi et al., 1990). Transformation of normal cells into tumoural ones also influences the activity of cathepsins. Cathepsin D in MCF-7 cells, secreted as a proenzyme, is more active in acidic pH than in normal mammary cells. The biological meaning of these differences is still unclear (Garcia et al., 1996).
Until now specific tissue inhibitors of cathepsins are unknown. However, it was documented that the propeptide of Cathepsin L selectively inhibits the activity of mature forms of Cathepsins K and L (Guay et al., 2000).

1.5.2 Physiological function of cathepsins

The function of Cathepsins S, V and K is tissue-specific. Cathepsin K is crucial for bone formation (Chapman et al., 1997, Turk et al., 2001). Although most cathepsins are ubiquitously expressed and implicated in generalised activities, some cathepsins (B, H, L, F, C, X, O) are involved in specialised processes. Cathepsins S and L participate in the processing of the MHC class II-associated invariant chain (Turk et al., 2001), crucial for immunosystem response or with periodic hair loss and hyperplasia (Turk et al., 2001, Roth et al., 2000). Cathepsins B, D and L were found to play a role in thyroglobulin processing (Dunn et al., 1991).

1.5.3 Trafficking of cathepsins

Most soluble lysosomal enzymes contain mannose-6-phosphate (M6P) residues, which are recognised by mannose-6-phosphate receptors (M6P-R) localised in the trans-Golgi network. Within this network they are transported to the endosomes rather than the lysosomes (Griffiths et al., 1986, von Figura et al., 1991).
Localisation of cathepsins in cells can differ depending on the cell type and conditions. In Cathepsin L-transfected fibroblasts inactive forms of this protein were found in dense cores within small vesicles and multivesicular endosomes labelled with endosome marker CD63 (Ahn et al., 2002) or in the nucleus during the G1 to S phase progression of cell cycle (Goulet, et al. 2004). Targeting of Cathepsin D is altered in several tumour cell lines, characteristic for increased Procathepsin D secretion. Higher pH in sorting endosomes prevents dissociation of M6PR-

cathepsin D complex, making recycling of the receptors impossible. Treatment of normal mammary cells with NH_4Cl led to medium secretion of newly synthesised Procathepsin D, whereas in many breast or ovarian cancer cells it is accumulated inside the cells (Garcia et al., 1996). In inflamatory responses cathepsins are released from the cells in elastine-rich tissues (Carmeliet et al., 1997, Chapman et al., 1997). The main elastases of this family are Cathepsins S, L and K (Erickson et al., 1989, McGrath et al., 1999).

1.5.4 Cathepsins in cancer

Asside from the physiological functions cathepsins are also involved in cancer development. They are upregulated in cancers of both epithelial and mesenchymal origin (breast, brain, lung, gastrointestinal, colorectal cancer) (Berdowska et al., 2004, Gocheva et al., 2007, Jedeszko et al., 2004) and in several cancers serve as diagnostic and prognostic factors. In lung, breast, ovarian, brain, head and neck cancer as well as in melanoma an increased expression of Cathepsin B is correlated with poor prognosis (Jedeszko et al., 2004), while for patients with breast, colorectal, head and neck cancers Cathepsin L is prognostic indicator of shorter survival rates (Berdowska et al., 2004). Patients suffering from breast cancer containing high levels of Cathepsins L or B in the primary tumours were at a higher risk of recurrence than those with low levels of both enzymes (Thomssen et al., 1995). These both cathepsins are also important players in malignancy progression of human pancreatic endocrine tumours (Gocheva et al., 2006). *In vitro* experiments employing weakly metastatic melanoma cell line describe both proteases as inducers of cells invasiveness (Goldmann et al., 1999, Szpaderska et al., 2001), and silencing of Cathepsin B in several carcinoma cell lines (Emmert-Buck et al., 1994, Krueger et al., 1999, Szpaderska et al., 2001) or downregularion of Cathepsin L in a glioma cell line (Levicar et al., 2003) reduced motility and invasiveness of the cells. *In vivo* experiments performed on RIP1-Tag2 mouse of pancreatic islet cell cancers reveal upregulation of Cathepsins B, C, H, L, S and X and their involvement in cancer development (Joyce et al., 2004). Active proteases were localised perinuclear or diffused, which suggests cell membrane-associated and/or ECM-distributed activity

significantly increased at the invasive edges of the islets carcinomas. An increase of the cysteine cathepsins activity was also observed in K14-HP/E2 mice bearing CIN-3 displasias or tumours, when in normal cervix no activity was detected (Joyce et al., 2004). Cathepsin D has been described as playing a role in metastatic processes. Experiments *in vitro* revealed that breast cancer cells created large intracellular acidic vesicles (LAVs) more frequently than normal mammary cells. Experiments with MDA-MB-231 breast cancer cells showed increased numbers of LAVs producing cells after matrix gel migration than before (Garcia et al., 1996). Breast cancer tumours overexpress this protease inside the cancer tissue but not in tumour fibroblasts or macrophags surrounding the tumour tissue (Garcia et al., 1996). Similar, the tumour cells, but not normal oesophageal epithelium cells adjacent to carcinomas, express Cathepsin D. Moreover, this expression is associated with invasive tumour characteristics and a poor prognosis for patients (Ikeguchi et al., 2002).

1.5.5 Cathepsins and relaxin

Relaxin and related peptides modulate activity of cathepsins. In breast cancer, relaxin induces the production and secretion of Cathepsin L and at the same time increases elastinolytic activity of the cells (Y. Radestock not published data). In pubic symphyses, relaxin in tandem with oestrogen increased activity of Cathepsin B (McDonald et al., 1982).

Our investigations on follicular thyroid carcinoma cells FTC-133 display increased levels of Cathepsin L and Cathepsin D, which coincided with elevated elastinolytic activity, induced by members of relaxin-like proteins INSL3 (Bialek et al., 2009). Higher Cathepsin L activity was also noticed in papillary thyroid carcinomas compared with normal adjacent thyroid tissue or normal thyroid from autopsies (Shuja et al., 1996), and in anaplastic thyroid carcinoma cell line 8505C compared with follicular thyroid carcinoma FTC-133 cell line (Plehn et al., 2000).

1.6 Thyroid gland

The thyroid gland (*Glandula thyreoidea*) is an endocrine gland found in the neck inferior to the thyroid cartilage (also known as the Adam's apple in men) and at approximately the same level as the cricoid cartilage. The shape of the thyroid is species dependent. In humans, it has a “butterfly” shape, where two lateral lobes are connected via the isthmus (Bem et al., 1995). Depending on gender, age and physiological state (menstrual cycle, pregnancy), as well as environment factors, it weighs between 15 and 30 g in humans (Bem et al., 1995).

1.6.1 Benign thyroid goiter

The term non-toxic goiter refers to enlargement of the thyroid which is not associated with overproduction of thyroid hormones or malignancy. Numerous cytokines and growth factors can affect thyroid function and this could account for the thyroid enlargement and growth of some nodules, which may be “hot” (take up radioactive iodine and show increased thyroid hormone synthesis) or “cold” (non-functional). Goiter is often merely a symptom of a more serious thyroid dysfunction such as:

- Hyperthyroidism, an overactive thyroid gland caused by:
    - Graves' disease (~80%) - autoimmune with stimulating antibodies to the TSH receptor
    - Toxic multinodular goiter (~ 15%)
    - Toxic adenoma (~ 2%)
    - Thyroiditis (~ 1%)
    - TSH secreting pituitary tumor (<0.01%)
    - Trophoblastic tumors (<0.001%)
    - Thyrotoxicosis factitia (<1%)
    - Thyroid follicular carcinoma (<0.01%)

- Hypothyroidism, an underactive thyroid gland caused mainly by:
    - Hashimoto's disease - autoimmune thyroid destruction
    - Primary (atrophic) hypothyroidism (probably endstage of Hashimoto's disease)
    - Post-radioiodine therapy which destroys thyroid tissue

- Post-surgery of the gland
- Thyroiditis (non-lymphocytic)
- Impaired T4 synthesis due to genetic defect
- Antithyroid drugs
- Loss of function TSH receptor mutations
- Thyroid hormone resistance

- Other forms of thyroiditis (De Quervain's or Riedel's thyroiditis)

1.6.2 Thyroid carcinoma

Tumours of the thyroid gland may be primary (arising from the cells within the thyroid gland) or secondary due to malignant cells which have spread from other tissues. The majority of primary tumours arise from epithelial cells of the thyroid gland and are therefore termed adenomas if benign and carcinomas if malignant.

Those arising from parafollicular cells (also termed C cells; produce hormone calcitonin) are called medullary thyroid carcinomas (MTC). About 25% of MTC is genetic in nature and is classified as familial MTC (caused by a mutation in the RET proto-oncogene). MTC which occurs by itself is termed as sporadic and when it coexists with tumours of the parathyroid gland and medullary component of the adrenal glands (pheochromocytoma) it is called multiple endocrine neoplasia type 2 (MEN2).

The epithelial cell tumours are sub-classified as papillary (PTC), follicular (FTC) or undifferentiated (UTC), according to their histological appearance. PTC and FTC are found more often in women (2 to 4 fold more often than men), aged 45–50 years. In areas with adequate iodine intake, the commonest tumour is papillary, accounting for some 80% of all tumours. Where iodine intake is low there is a relative increase in follicular and anaplastic carcinoma, though no overall increase in frequency. The prognosis of PTC is usually optimistic with long-term survival rates of more than 90%. FTC predicts more aggressive behaviour with recurrences or/and distant metastases to liver, lung and bones. Undifferentiated thyroid tumours are much less common, metastasize early, and have a much poorer prognosis with a 5-years survival rate lower than 5%. A number of factors, both genetic and environmental have been implicated in the etiology of epithelial tumours (Nussey et al., 2001).

Etiological factors in thyroid cancer:

Growth factors: the role of such known thyroid growth factors as TGF-α, EGF, VEGF and IGF-1 in neoplasia remains uncertain.

Oncogenes: **RET** is a gene coding for a tyrosine kinase receptor for neurotrophic growth factor. It is not normally expressed in thyroid follicular cell tumours. Rearrangements of this gene are frequently associated with papillary thyroid carcinoma and occur as a fusion of tyrosine kinase domain of RET to 5' portion of other gene. In consequence the MAPK pathway, important in PTC development, is constitutively active (Fiore et al., 2009). The rearrangements of RET are particularly seen in patients who have had tumours after irradiation e.g. papillary tumours post-Chernobyl. There are at least 10 forms of Ret oncogenes which have been designated as RET/PTC1, RET/PTC2, RET/PTC3…RET/PTC10 (where PTC stands for papillary thyroid carcinoma). Ret is also a factor in medullary cell carcinoma of the thyroid gland.

RAS is a membrane associated monomeric G protein involved in signal transduction processes. Activating mutations of RAS genes are found with a similar frequency in follicular adenomas and carcinomas.

p53 is a tumour-suppressor gene. Inactivating mutations of p53 are seen in about 10% of thyroid carcinomas and mainly in undifferentiated thyroid carcinoma (Malaguarnera et al., 2007, Olivier et al., 2002)

Thyroid irradiation: external irradiation dose-dependently increases the incidence of thyroid cancer and is marked in younger patients. Therapeutic doses of radioiodine do not appear to result in an increased risk of thyroid malignancy (Boltze et al., 2002, Reiners et al., 2008, Tronko et al., 2006).

Other: familial cases of thyroid cancer have been reported in familial adenomatosis coli, Gardner's disease and Cowden's syndrome. There is controversy over the association with certain histocompatibility antigens (Riesco-Eizaguirre et al., 2007).

1.6.3 Prognostic factors in thyroid carcinoma

One important prognostic factor for differentiated thyroid carcinoma is a patient's age. Younger patients (under 40) usually have better prediction (Grant et al., 1988). Lack of extracapsular extension or vascular invasion increases the chances for a good prognosis (Grant et al., 1988, Lennard et al., 2001, Mazzaferri et al., 1994, Sanders et al., 1998, Stauton et al., 1994).

Improved understanding of the genetic events associated with thyroid carcinogenesis and progression to more aggressive forms may lead to the identification of more reliable tumour-specific prognostic markers. Currently it is thought that PTC and FTC arise independently of one another, whereas there is some evidence to suggest a progression from FA to FTC (Puxeddu et al., 2001).

Molecular events common in patients with PTC, particularly those exposed to ionizing radiation, are genomic rearrangements that result in RET proto-oncogene activation (Nikiforov et al., 2008). Mutations in RAS have also been detected in PTC, but are more commonly detected in FA and FTC, and have been thought to be among the earliest events in cancer progression. More recently somatic mutation of the BRAF gene has been found to be an even more common genetic event accompanying the development of PTC (Puxeddu et al., 2008). Current evidence indicates that mutations in RAS, BRAF and RET do not overlap with PTC. Recent studies indicate that chromosomal rearrangement, frequent in FA, which results in the fusion of genes between the thyroid-specific transcription factor PAX8 (2q13) and the PPARγ (3p25), may be involved in FA to FTC progression (Castellone et al., 2008, Puxeddu et al., 2001, 2008, Nikiforov et al., 2008).

Improved understanding of the molecular events associated with thyroid carcinogenesis and progression to more aggressive forms may lead to the identification of more reliable tumour-specific prognostic markers. Investigations performed in our group demonstrated that CD97, a dimeric glycoprotein belonging to the secretin receptor superfamily, might play an important role in the dedifferentiation of thyroid tumours. In normal thyroid tissue no CD97 immunoreactivity could be found, whereas in differentiated thyroid carcinomas CD97 expression was either lacking or low. Undifferentiated thyroid carcinomas revealed high CD97 expression (Aust et al., 1997, Hoang-Vu et al., 1999). Useful markers for thyroid carcinoma differentiation and progression include different expressions and activities of proteins

such as telomerase, E-cadherin, maspin, APN and PPARgamma (Aldred et al., 2003, Boltze et al., 2003, Brabant et al., 1993, Hoang-Vu et al., 2002, Kehlen et al., 2003, Scheumman et al., 1995).

More recent findings also demonstrate that the Raf-1 kinase inhibitory protein (RKIP) and ENO1 are proteins that could be involved in thyroid tumourigenesis. RKIP is a predictive factor for thyroid carcinoma patients with lymph node and distant metastasis (Trojanowicz et al., 2008). Pre-treatment of follicular thyroid carcinoma cells with retinoic acid decreased the proliferation and re-differentiation rate, reduced ENO1 expression and decreased invasive abilities (Trojanowicz et al., 2009).

Benign or Early Stage Thyroid Carcinoma Markers	**Early and Late Stage Thyroid Carcinoma Markers**
Thyroid Peroxidase (TPO)	RET/PTC
Thyroglobulin (Tg)	RAS
TSH Receptor (TSHR)	BRAF
Na Iodide Symporter (NIS)	PAX8/PPARγ
TTF-1	Mucin (Muc1)
	Proliferating Cell Nuclear Antigen (PCNA)
	Leu-M1 Antigen
	p53
	DNA methylase
	Telomerase
	Focal Adhesion Kinase (FAK)
	Galectin-3
	Ki-67 (MIB1)
	Oncofetal Fibronectin

Table 1: Markers that have been studied for the detection of benign and malignant thyroid cancers.

2 Aim of the work

The aim of this work was to investigate the function of relaxin 2 in thyroid carcinoma. The hormone was found to be increased in thyroid carcinoma tissues compared with benign tissues. All samples investigated revealed also the expression of receptors for relaxin 2 suggesting that thyroid gland could be a possible target organ for relaxin 2 actions.

In order to investigate the possible role of relaxin 2, we generated stable transfectants expressing this hormone. Using this approach, we studied the proliferation, motility, and matrix-invasive potential of follicular thyroid carcinoma cells. Moreover, we aimed to characterise the alternations in morphology of the transfectants, protein expression and proteases activity, which are the main consideration points to understand the role of relaxin 2 in thyroid carcinoma progression.

3 Materials and methods

3.1 Materials

3.1.1 Chemicals and reagents

Buffers	Contents
	Cell culture
Phosphate buffered saline (PBS)	137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4 x 7 H_2O; 1.4 mM KH_2PO_4; pH 7.4
Hank's Balanced Salts (HBSS)	Gibco/Invitrogen
	RNA/PCR
10xTBE	890 mM Tris-Base; Boric acid; 20 mM EGTA; pH 8.0
10x PCR Buffer	Amersham/GE Heatlhcare
10x Taq-Gold Buffer	Amersham/ GE Heatlhcare
RNA measuring buffer	0.1%TRIS/HCl (1 M) pH 7.5 in DEPC H_2O
	Protein
5x Laemmli lysis buffer	1 M TRIS pH 6.8, 10% SDS, 50% Glycerol, 10% Mercaptoethanol, 5% Bromophenol blue
Native whole cell lyses buffer	20 mM HEPES (pH 7.4), 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM DTT
Loading buffer	0.5 M TRIS/HCl pH 6.8, Glycerin, 10% SDS, Bromophenol Blue, Aqua bidest
Native loading buffer	0.5 M TRIS/HCl pH 6.8, Glycerin, Bromophenol Blue, Aqua bidest
Tris/HCl buffers	50 mM Tris/HCl; pH 7.5;
Western-blot running buffer (10x)	3% TRIS, 14.4 Glycine, 0.6% SDS
Western-blot transfer buffer	1.4% Glycine, 0.3% TRIS, 20% Methanol
TBS(T)	10 mM Tris-Base; 0.5 M NaCl; pH 7.5 + 0.1% TWEEN20
PBS(T)	PBS + 0.1% TWEEN20
Stripping solution	0.2 M Glycine pH 2.5, 0.05% Tween 20
SDS wash-out buffer	2.5% Triton X-100 in bidestillated H_2O
Proteases activation buffer	50 mM Tris/HCl; pH 7.5; 200 mM NaCl; 10 mM $CaCl_2$; 1 µM $ZnCl_2$
2D protein lysis buffer	42% Urea; 15% Thiourea; 4% CHAPS; 0.6% DTT; 500 µl/ 100 ml Pharmalyte
Rehydration buffer	7 M Urea, 2 M Thiourea, 4% CHAPS, 0.5% Pharmalyte, 40 mM DTT
2D anode buffer	0.025 M TRIS, 0.2 M Glycine, 0.002 M SDS
2D cathode buffer	0.05 M TRIS, 0.4 M Glycine, 0.007 M SDS
Equilibrations buffer (stock)	6 M Urea, 2x SDS, 50 mM TRIS/HCl pH 8.8, 30% Glycerine, 500 µl Bromophenol Blue
IAA Equilibrations buffer	1% DTT in Equilibrations buffer (stock)
DTT Equilibrations buffer	2.5% IAA in Equilibrations buffer (stock)
2D Fixation buffer	40% Ethanol, 10% Acetic Acid
2D Sensitizing buffer	0.2% Sodiumthiosulfate
2D Stopping buffer	5% Acetic Acid
MS Protein analysis buffer	50% ACN/ 0,1% TFA
Saponin Buffer	2% BSA, 0,05% Saponin, 0.1% Sodiumacide in PBS
Bouin fixative	750 ml Picrinic acid, 250 ml 37% formaldehyde, 50 ml Galcial acetic acid

Substances	Origin
	Cell culture
G418- Sulfate (Geneticine)	Invitrogen, Karlsruhe, Germany
HCl	VWR, Darmstadt, Germany
Natrium Carbonate	Merck, Darmstadt, Germany
Elastin, soluble from human lung	Sigma-Aldrich, Steinheim, Germany
IBMX	Sigma-Aldrich,
PBS	AppliChem GmbH, Darmstadt, Germany
Forskolin	Calbiochem/Merck, Darmstadt, Germany
Relaxin	Phoenix Europe GmbH, Karlsruhe, Germany
GM6001 (Ilomastat) MMP Inhibitor	Chemicon/Millipore GmbH, Schwalbach
Non-silencing siRNA	Qiagen, Hilden, Germany
LGR7 siRNA	Qiagen,
MMP-2/ MMP-9 Substrate	Calbiochem/Merck, Darmstadt, Germany
3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide	Sigma-Aldrich,
Natrium Carbonate ($NaHCO_3$)	Merck
Lipofectamine 2000	Invitrogen
Fetal Calf Serum (FCS)	BioWest, Nuaille, France
Natrium Carbonate	Merck
Trypsin/EDTA	Gibco/Invitrogen, Karlsruhe, Germany
Collagen A	Biochrom AG, Berlin, Germany
Gelatine	Biochrom AG
	RNA/PCR
Trizol reagent	Invitrogen
Chlorophorm	MERCK
Isopropyl alcohol	Merck
Ethanol	MERCK
RNAse free Water	Qiagen
RNAse out	Invitrogen
Random primer	Invitrogen
AmpliTaq polymerase -Gold	Roche, Penzberg, Germany
Taq polymerase	Amersham/GE Healthcare, München, Germany
Agarose	Roche
Ethidium Bromide	Serva, Heidelberg, Germany
peQ Universal Agarose	PeQLab Biotechnology, Erlangen, Germany
100 bp DNA Ladder	Invitrogen
1000 bp DNA Ladder	Invitrogen
	Protein
Acrylamide	Roth, Karlsruhe, Germany
TRIS	Amersham/GE Healthcare
HCl	VWR,
PlusOne™ SDS	Amersham/GE Healthcare
Ammoniumpersulfate (APS)	Pharmacia Biotech, Freiburg, Germany
TEMED	Biorad, München, Germany
Glycine	Serva,
TWEEN 20	Serva

Substances	Origin
Broad range protein marker	Promega, Mannhein, Germany
High-Range Rainbow Molecular Weight Markers	Amersham/GE Healthcare
Bovine Serum Albumin	Sigma-Aldrich
Milk powder	Sucofin
X-ray film (Hyperfilm)	Amersham/GE Healthcare
EDTA	MERCK
Protease inhibitor cocktail	Roche
Acrylamide	Amersham/GE Healthcare
Pharmalyte	Amersham/GE Healthcare
PlusOne™ SDS	Amersham/GE Healthcare
PlusOne™ TEMED	Amersham/GE Healthcare
PlusOne™ Glycine	Amersham/GE Healthcare
CHAPS	Amersham/GE Healthcare
Cleaning solution	Amersham/GE Healthcare
Dithiothreitol (DTT)	Roth
HCl	VWR
Hydrogen peroxide solution, 30%	Merck
Isopropyl alcohol	Merck
Iodoacetamide	Sigma-Aldrich
Methanol	VWR
Natrium Carbonate	Merck
Natrium sulfate	Roth
Urea	Amersham/GE Healthcare
Triton X-100	Sigma-Aldrich
Acidic acid	VWR
Donkey serum	Dako, Glostrup, Denmark
Goat serum	Dako
Xylol	Roth
Isopropanol	Merck
Picrinic acid	Sigma-Aldrich
Paraffin	Engelbrecht Medizin- und Labortechnik GmbH, Edermünde, Germany

Staining Solutions	Origin
Toluidin blue solution	Amersham/GE Healthcare
Coomassie Blue R250	Amersham/GE Healthcare
Mayer's Hemalaun	Merck
Eosine	Merck
Bromophenol Blue	Amersham/GE Healthcare
Silver nitrate solution	Roth

Medium in cell culture	Origin
Luria-Broth Medium	Gibco/Invitrogen
D-MEM/F12-medium	Gibco/Invitrogen
OPTIMEM	Gibco/Invitrogen

Antibodies	Origin
R6 Relaxin	Prof. Bernhard Steinetz, Nelson Institute of Enviromental Medicine, New York University Medical Center, Tuxedo, NY
Anti-Relaxin 2 Rabbit pAb (553850)	Calbiochem/Merck
TIMP1	Oncogene Research Products/Calbiochem/Merck, Darmstadt, Germany
TIMP2	Santa Cruz Biotechnology, Santa Cruz, USA
Anti-TIMP3 Mouse mAb (136-13H4)	Calbiochem/Merck
TIMP4	Santa Cruz Biotechnology
MMP-1	Santa Cruz Biotechnology
MMP-2	Oncogene Research Products/Calbiochem/Merck, Darmstadt, Germany
MMP-9	Santa Cruz Biotechnology
MT1-MMP (Ab-1)	Calbiochem/Merck
Cathepsin L 33/1	Prof. E. Weber MLU Halle/Saale
Cathepsin D	Prof. E. Weber MLU Halle/Saale
Procathepsin L 2D4	Prof. E. Weber MLU Halle/Saale
Cathepsin B	Prof. E. Weber MLU Halle/Saale
Cathepsin K CK641C7	Prof. E. Weber MLU Halle/Saale
Cathepsin H	Prof. E. Weber MLU Halle/Saale
ADAM23	USBiological/Biomol, Hamburg, Germany
ADAMTS-1	Abcam, Cambridge, UK
ADAMTS-5	Abcam
Fluorescent Deoxyribonuclease I Conjugates	Invitrogen/Molecular Probes, Karlsruhe, Germany
Rhodamine-phalloidin	Invitrogen/Molecular Proges
Alpha-tubulin	Sigma-Aldrich
Acetylated-tubulin	Sigma-Aldrich
Tyronisated-tubulin	Sigma-Aldrich
Polyglutaminated-tubulin	Sigma-Aldrich
Cofilin1	Cell Signalling Technology, Danvers, USA
Phosphocofilin1	Cell Signalling Technology
Collagen, Type III (Ab-1)	Oncogene Research Products/Calbiochem/Merck, Darmstadt, Germany

Kits	Origin
cAMP	Amersham/GE Healthcare
BrdU assay	Roche
RLNelisa	Immundiagnostik AG, Benshein, Germany
2-D Quant Kit	Amersham/GE Healthcare
Sample Grinding Kit	Amersham/GE Healthcare
Pierce Western Blotting substrate Reagents	Perbio Science/Thermo Scientific, Bonn, Germany
LSAB-Kit-plus	Dako
Kodak developing solution	Kodak, Rochester, USA

3.1.2 Instruments

Cell culture	Source
Cell incubator, Herasafe	Heraeus Holding GmbH, Hanau, Germany
Safety cabinet, HS 12	Heraeus Holding GmbH
Cool centrifuge, Hettich POTANTA/RP	Heraeus Holding GmbH
Water bath box, WB14	Memmert GmbH, Schwabach, Germany
Light (Fluorescence) Microscope, Axiovert 25	Karl Zeiss, Jena, Germany
Plastic flasks	Greiner Bio-One, Solingen-Wald, Germany

RNA/PCR	Source
Horisontal Gelelectrophorese system	Bio-Rad Laboratories GmbH, München, Germany
Homogenizer (MICRO-DISMEMBRATOR S)	B. Braun Biotech international/Sartorius AG, Göttingen, Germany
3 TRIO-Thermocycler	Biometra, Göttingen, Germany
UV-Transluminator	Biometra
Table microcentrifuge	Denver Instrument, Göttingen, Germany
Eppendorf-Thermomixer 5436	Eppendorf AG, Hamburg, Germany
HIGH-SPEED-centrifuge	Heraeus Holding GmbH
Kodak scan camera, Image station 440 CF	Kodak

Protein	Source
Strip Holders	Amersham/GE Healthcare
Ettan IPGphor II IEF System	Amersham/GE Healthcare
Ettan DALTsix Electrophoresis Unit	Amersham/GE Healthcare
Voyager DE PRO MALDI-TOF workstation	Applied Biosystems, Darmstadt, Germany
PALM MicroLaser system	P.A.L.M. Microlaser Technologies/Karl Zeiss, Jena, Germany
Cryotom (HM 560)	Microm AG, Volketswil, Switzerland
Trans-Blot Cell	Bio-Rad Laboratories GmbH
Mini-Protean II device	Bio-Rad Laboratories GmbH
WK230 LAUDA cooling system	Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim, Germany

Instruments	Source
Spectrophotometer UV 1602	Shimadzu, Duisburg, Germany
Spectra Rainbow ELISA	Tecan, Crailsheim, Germany
Precision balance	Sartorius AG, Göttingen, Germany
pH-meter	WTW Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany
Speedvac	Eppendorf AG

3.1.3 Cell lines

Cell type	Appellation	Reference
Thyroid	FTC-133	supplied by Prof. P. Goretzki, established from a lymph node metastasis of a follicular thyroid carcinoma from a 42-year-old male; 90% DMEM/F12+ 10% FBS
	FTC-236	supplied by Prof. P. Goretzki , established from a lymph node metastasis of a follicular thyroid carcinoma, from which the FTC 133 cell line had been established, 90% DMEM/F12+ 10% FBS
	FTC-238	supplied by Prof. P. Goretzki, established from a lung metastasis of a follicular thyroid carcinoma from a 42-year-old male; 90% DMEM/F12+ 10% FBS
	BC-PAP	DSMZ, Braunschweig, Germany established from the tumour tissue of a 76-year-old woman with metastasizing papillary thyroid carcinoma, 90% RPMI 1640 + 10% FBS
	C-643	established from undifferentiated thyroid carcinoma; 90% DMEM/F12+ 10% FBS
	HTh74	established from undifferentiated thyroid carcinoma; 90% DMEM/F12+ 10% FBS
	8305C	DSMZ, Braunschweig, Germany established from undifferentiated thyroid carcinoma of a 67-year-old woman; 90% DMEM/F12+ 10% FBS
	8505C	DSMZ, Braunschweig, Germany, established from undifferentiated thyroid carcinomas of a 78-year-old woman; 90% DMEM/F12+ 10% FBS

Table 2: Cell lines and cell culture media used in this study

3.1.4 Tissues

Thyroid tissue specimens from 59 patients were investigated in the present study. Tissues of all patients had been obtained after surgery performed between 1994 and 2001 at the Department of General, Visceral and Vascular Surgery, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany. Tumour tissues were staged according to the Tumour-Node-Metastasis (TNM) staging classification (UICC-AJCC 1997). The specimens were cryopreserved in liquid nitrogen after resection. The study was approved by the ethical committee of the Martin Luther University, Faculty of Medicine, and all patients gave written consent.

Tissue	Gender	Age	PTNM
PTC (*n* = 14)	M	25	T4aN1bM1
	F	51	T4N1aM0
	F	56	T4N1bM1
	F	14	T4N0M0
	F	14	T4N1M0
	M	65	T3N1Mx
	F	71	T3N1M1
	M	11	T3N1Mx
	M	36	T2N1Mx

Tissue	Gender	Age	PTNM
	M	63	T2aN0M0
	F	27	T2N1aMx
	F	59	T1N0M0
	F	39	T1N0M0
	F	55	T1aN0M0
FTC (*n* = 12)	F	53	T4N0M0
	F	60	T4NxM1
	F	62	T4N1bM1
	F	34	T4N0M0
	F	60	T4N0M0
	M	60	T4NxM2
	F	51	T3N1bM0
	M	67	T3bN1bM1
	M	43	T3N0M0
	M	63	T3N0M0
	F	46	T3NxM0
	F	54	T2NxMx
UTC (*n* = 14)	F	79	T4N2Mx
	F	72	T4N2Mx
	F	76	T4N1Mx
	F	58	T4N1Mx
	F	70	T4N1aMx
	M	67	T4N1Mx
	F	87	T4N0M1
	F	53	T4N0M1
	F	75	T4N0M1
	F	68	T4N0M1
	F	42	T4N0M1
	M	66	T4N0M0
	F	69	T3NxMx
	M	52	T3N0M0

Table 3: List of thyroid carcinoma tissues used in these studies; PTC –papillary thyroid carcinoma, FTC- Follicular thyroid carcinoma, UTC- undifferentiated thyroid carcinoma; M – male, F - female

3.2 Methods

3.2.1 Culture of human thyroid carcinoma cells

Adherent FTC-133 and FTC-238 cells were grown in small flasks (25 cm^2) until 80% confluency, than washed with 10 ml HBSS. 2 ml of Trypsine/EDTA solution was added to the flask and incubated 2-5 min in 37℃ humidified incubator. After detaching from the bottom, the cells were collected and centrifuged at 1500 rpm for

5 min. The pellet was suspended in 5 ml 10% FCS DMEM/F12 medium. Cells were counted in a Neubauer chamber and seeded at $1x10^4$ cells per small flask in 7-10 ml medium for further culturing. The culture medium was changed every 2-3 days. For experiments, $1x10^4$ cells were seeded in six-well plates, $1x10^5$ in small-size flasks, $5x10^5$ in middle-size flasks and $1x10^6$ in big-sized flasks (75 cm^2) in serum-free medium.

3.2.2 Cell freezing and thawing

Cells from 80% confluent big flasks were trypsinised, centrifuged and counted. $5x10^6$ cells were resuspended in 1 ml freezing medium (Fetal Calf Serum and DMSO; 1:9). Such prepared cells were sequentially and slowly frozen in -20°C for 24 h, then in -80°C for 24 h and finally stored in liquid nitrogen. Cells were defrosted in pre-warmed culture medium, centrifuged in 15 ml Falcon tubes, and supernatant was aspirated. The pellet was resuspended in fresh culture medium in a culture flask (25 cm^2).

3.2.3 Cryo- and paraffin-embedded tissues

Dissected mouse tissues and human thyroid tissues were snap frozen in liquid nitrogen and stored at -80°C until use. Tumours and organs collected from laboratory mice were also fixed in Bouin fixative. The next day, the tissues were washed extensively in 70% EtOH. For paraffin-embedding, tissues were incubated two times each in 70% EtOH, 96% EtOH, and iso-propanol, and 1x in Xylol, before paraffin-embedding.

Paraffin blocks with tumours were cut into 5 µm sections and one section was stained with haematoxylin and eosin.

3.2.4 RNA extraction from tissues and cells

Total RNA from homogenised cryotissues and cells was isolated using TRIZOL reagent according to the manufacturer's instructions. 1 ml of TRIZOL reagent was added to 100 mg of homogenised frozen tissue powder or directly to the culture flask. To lead the nucleoprotein complexes to destruction and avoid contamination with proteins, samples were incubated with TRIZOL at RT. After 5 min, 0.2 ml chloroform was added and each sample was shaked by hand and incubated for 2-3 min at RT.

Following centrifugation at 12000 x g at 4°C for 15 min, the upper phase containing the RNA was transferred into the new tubes. The remaining lower-phenol and interphase comprised DNA, proteins and salts. The RNA was precipitated with isopropanol (0.5 ml per 1 ml initial TRIZOL volume), incubated for 10 min at RT and centrifuged at 12000 x g at 4°C for 10 min. The supernatant was removed and remaining RNA pellet was washed with 1 ml 75% EtOH and centrifuged at 12000 x g at 4°C for 5 min. The washing step was repeated twice. After this procedure, the pellet was air-dried, resuspended in RNAse-free water at 55°C for 5 min and stored at -80°C. Total RNA from transfected cells for micro-array analysis was isolated with RNeasy extraction kit according to the manufacturers' instructions. RNA concentration was measured using a spectrophotometer at wave lengths between 260 and 320 nm.

3.2.5 RT-PCR analysis

Total RNA was used as a template for first strand cDNA synthesis, employing a Superscript reverse transcriptase kit and 500 ng/ml of oligo d(T) primers. 1 µg of RNA was diluted in DEPC-water to 10 µl end volume and denaturated in 95°C for 3 min. To such prepared RNA, 15 µl reaction mix (2.7 µl DEPC-water, 5.0 µl 5x First Strand Buffer, 2.5 µl 0.1 M DTT, 3.0 µl Random primers, 1.0 µl 12.5 mM dNTP, 0.3 µl superscript II and 0.5 µl RNase out) was added, mixed and incubated at 42°C for 45 min and 95°C for 3 min. The samples were stored at -20°C.

PCR reaction was performed with 25 µl solution containing 16.8 µl dH_2O, 2.5 µl 10x PCR buffer, 3.0 µl dNTP mixture (100 µM), 0.25 µl sense primer (10 pmol/ml), 0.25 µl antisense primer (10 pmol/ml), 0.2 µl polymerase (AmpliTaq – 5 U/µl, KlenTaq, TaqGold) and 2 µl cDNA sample. The amplification program was performed depending on target gene. Specific conditions are listed below in Table 4.

Primer	Primer sequence	bp	Cycle nr	T_M (°C)	Times (s)	Polymerase
Forward RLN2	5'-CGGACTCATGGGATGGAGGAAG-3'	222	35	61	30/30/30	Taq
Reverse RLN2	5'-GCTCCTGTGGCAAATTAGCAAC-3'					
Forward huLGR7	5'-CCCAATTCTCTATACTCTGACCACAAG-3'	243	40	65	60/120/60	TaqGold
Reverse huLGR7	5'-TCATGAATAGGAATTGAGTGTCGTTGATT-3'					
Forward 18S	5'-GTTGTTGGAGCGATTTGTCTGG-3'	344	15	62	30/30/30	Taq
Reverse 18S	5'-AGGGCAGGGACTTAATCAACGC-3'					

Table 4: Oligonucleotide primers used for determination of defined length (bp) fragments of genes at melting temperature (T_M) using one of two polymerases.

3.2.6 Agarose gel electrophoresis

Amplificons were visualised on 2% agarose gel containing ethidium bromide. The gels were photographed with Kodak Image System 440c. Semi-quantitative analyses of PCR gels were performed after normalising with 18s using Kodak Digital Science 1D-software (Kodak Digital Science Electrophoresis Documentation and Analysis System 120).

3.2.7 Total protein extraction and western blot analysis

Total cell lysates for western blotting were isolated using 1x reduced Laemmli buffer containing protease inhibitor cocktail. Analysis of proteins secreted to the serum-free, or 2.5% FCS supernatants, required centrifugation at 4000 x g. The pellet containing cells and insoluble elements were removed and supernatant with proteins secreted to the medium was placed into the new tubes and concentrated with speedvac. Protein concentration was measured using the Bicinchoninic Acid method, according to the manufacturers' instructions. Aliquots of 20 µg proteins were prepared and mixed at 1:1 with reduced 1x Laemmli buffer containing proteases inhibitors. The samples were then denaturated at 95°C for 5 min.

Proteins were resolved on polyacrylamide-SDS gels (SDS-PAGE). A 12% gel was used to separate proteins at molecular weight between 20 and 50 kDa; lower proteins were loaded on 15% and bigger on 10% gel. 5% stacking gel was joined to the upper edge of the separating gel. 20 µl proteins were loaded to each lane of the gel. To determine the size of proteins, a Broad Range Protein Marker was run in a separate lane of the gel. Electrophoresis was performed at 40 mA for about 2 h at RT. Proteins were transferred onto nitrocellulose membranes in wet mini-Transblot

cell at 17 V overnight or for 2 h at 1 A, both at 6°C, and stained with Ponceau staining solution. Proteins were blocked for 1 h at RT and incubated overnight at 4°C with specific antibody diluted in defined blocking buffer (Table 4). After washing with wash-buffer, membranes were incubated with horseradish peroxidase conjugated secondary antibody for 1 h, washed with wash-buffer and dipped in an immunodetection ECL kit for 1 min. Immunoreactive bands were visualised by exposing X-ray film and developed using Kodak detection kit. Densitometric data were obtained using Kodak Digital science 1D software.

3.2.8 Immunohistochemistry and immunocytochemistry

Paraffin blocks containing thyroid tissues or tumours obtained from *in vivo* mice experiments, were cut as 5 µm sections, dewaxed, rehydrated and stained with hematoxylin-eosine solution to determine morphology of the cells.

Incubation of sections with antibody required earlier dewaxing by warming in higher temperature and then rehydration in PBS-T (PBS with 0.1% Tween 20) followed by proteinase K treatment (30 µg/ml) for 30 min at 37°C and 3% MetOH for 25 min in RT to inactivate endogenous peroxidase activity. After washing in PBS-T, non-specific binding sites were blocked with 10% normal goat serum in PBS-T for 1 h at RT. RLN2 was detected using two previously mentioned antibodies. The first one, rabbit polyclonal antiserum against RLN2 obtained from Calbiochem/Merck, was diluted at 1:300 in blocking buffer and applied overnight at 4°C. The second antibody, R6 rabbit anti-porcine relaxin antiserum (generously provided by Prof. Bernhard Steinetz, Nelson Institute of Environmental Medicine, New York University Medical Centre, Tuxedo, NY), was diluted 1:800 in blocking buffer overnight at 4°C. As a control, non-immune rabbit serum was used. The second antibody, horseradish peroxidase conjugated goat-anti-rabbit antibody, was used at 1:500 in PBS-T at RT for 1 h. Detection was performed with filtrated diaminobenzidine solution for 10 min and haematoxylin.

Semi-quantitative planimetric measurement was performed using Axioplan light microscope and Zeiss KS300 software. The immunostained area was compared with the total section area, defined as 100%, and calculated as a percentage ratio. Sections were classified as negative when expression was 10% or below, low

expression – 11-14%, moderate expression – 40-80% and high expression – more than 80%.

Immunocytochemistry was used to define the expression of proteins MMP-2 and TIMP1. The defined number of cells ($1x10^4$ cells/ml) was dropped onto sterile glass slides and grown in Petri dishes for 3-4 days, changing the medium everyday. Cells were washed with PBS and fixed for 20 min with a mixture of 3% H_2O_2 in ice cold 90% MetOH 1:4. After washing in PBS, non-specific bindings were blocked by incubation with normal swine serum diluted 1:4 with 1% PBS-BSA for 10 min. Mouse monoclonal antibodies against TIMP1 and MMP-2 were each diluted 1:20 in saponin buffer and applied to the sections overnight at 4°C. Negative controls were incubated with saponin buffer only. After washing in PBS, slides were incubated with secondary goat-anti-mouse antibody diluted at 1:20000 with PBS, followed by treating with an avidin-biotin-peroxidase complex. Immunopositive staining was visualised using diaminobenzidine (DAB) chromogenic solution (1:50) and contrastained with Mayer's haematoxylin.

3.2.9 Immunofluorescent staining

The defined number of cells ($1x10^4$ /ml) were seeded on the glass slides and grown for 3-4 days, changing the medium everyday. After washing with PBS, cells were fixed for 20 min in 3.7% paraformaldehyde (PFA). Followed by blocking of non-specific binding with defined block buffers, slides were then incubated overnight at 4°C with specific antibodies diluted in saponin buffer (Table 5). The negative controls were incubated in saponin buffer only. A secondary rhodamine-bounded antibody, specific to the first, was applied in PBS-T at a dilution of 1:20000 for 1 h at RT. Nuclei were stained with Hoechst staining solution diluted at 1:100 in PBS-T for 1 min. For viewing the fluorescence, cells were covered with Mounting Medium. Every step in this assay was preceded by washing the cells in PBS-T 0.1%.

Fluorescent staining of F and G actin was performed using rhodamine labelled first antibodies diluted at 1:100 and 1:50 respectively in saponin buffer. The further procedure was followed as written before, but omitting the secondary antibody incubation. Pictures were taken using fluorescent microscopy or confocal laser-scanning microscopy.

Antibody	Size of protein	Dilution in Western blot	Secondary Antibody
R6 Relaxin	Matured - 6 kDa Proform – 18 kDa	1:2500 in 5% BSA1% Milk in PBST	1:20 000 GAR
TIMP2	Matured – 21 kDa	1:500 in BSA in PBST 0.1%	1:20 000 AG
TIMP3	Unglycosylated – 21-24 kDa Glycosylated – 30 kDa Duplex – 48 kDa	1:850 in 3% BSA in TBST 0.1%	1:20 000 GAM
TIMP4	Matured – 28 kDa	1:850 in 5% BSA in PBST 0.1%	1:40 000 AG
MMP-2	Matured – 62 kDa Proform – 72 kDa	1:500 in 5% milk in PBST 0.1%	1:20 000 GAM
MT1-MMP (Ab-3)	Matured – 60 kDa Proform – 57 kDa	1:1000 in 3% BSA in PBST 0.1%	1:20 000 GAM
Cathepsin L 33/1	Single chain – 24 kDa Heavy chain – 31 kDa Proform – 42 kDa	1:500 in 5% milk in PBST 0.1%	1:20 000 GAM
Cathepsin D	Proform – 52 kDa Matured – 38 kDa	1:5000 in 5% milk in PBST 0.1%	1:20 000 GAR
Procathepsin L 2D4	Proform – 42 kDa	1:500 in 5% milk in PBST 0.1%	1:20 000 GAM
Cathepsin B	Matured – 25 kDa	1:200 in 5% milk in PBST 0.1%	1:20 000 GAR
Cathepsin K CK641C7	Matured – 25 kDa	1:200 in 5% milk in PBST 0.1%	1:10 000 Anti IGG+IGM
Cathepsin H	Matured – 50 kDa	1:10 000 in 5% milk in PBST 0.1%	1:20 000 GAR
ADAM23	Preproform – 92 kDa Proform – 74 kDa Matured – 60 kDa	1:1000 in 3% BSA in PBST 0.1%	1:20 000 GAR
ADAMTS-1	Zymogen – 110-120 kDa Matured – 84-98 kDa Breakdown products – 34-50 kDa	1:850 in 1% milk in PBST 0.1%	1:20 000 GAR
ADAMTS-5	Zymogen – 120 kDa Matured – 73 kDa Breakdown products – 40-50 kDa	1:2500 in 1% milk in PBST 0.1%	1:20 000 GAR
Cofilin1	19 kDa	1:1000 in 5% milk in TBST	1:20 000 GAR
Phosphocofilin1	19 kDa	1:1000 in 5% milk in TBST	1:20 000 GAR
Cdc42	21 kDa	1:1000 in 5% milk in TBST	1:20 000 GAR

Antibody	Dilution in immunocytochemistry	Secondary Antibody
INSL3 serum	1:200 in Saponin Buffer	1:300 TRITC GAR
TIMP1	1:10 Medium (DAKO)	DAKO LSAB Kit
MMP-1	1:10 Medium (DAKO)	DAKO LSAB Kit
MMP-2	1:10 Medium (DAKO)	DAKO LSAB Kit
MMP-9	1:10 Medium (DAKO)	DAKO LSAB Kit
Cathepsin L 33/1	1:100 in Saponin Buffer	1:100 Rhodamine DAM
Cathepsin D	1:100 in Saponin Buffer	1:100 TRITC GAR
Procathepsin L 2D4	1:100 in Saponin Buffer	1:100 Rhodamine DAM
Rhodamine- phalloidin	blocking – 3% milk in PBST Ab - 1:100 in 3% BSA in PBST	-
Fluorescent Deoxyribonuclease I Conjugates	1:50 in Saponin Buffer	-
Alpha-tubulin	1:100 in Saponin Buffer	1:100 Rhodamine DAM
Acetylated-tubulin	1:50 in Saponin Buffer	1:100 Rhodamine DAM
Tyrosinated-tubulin	1:100 in Saponin Buffer	1:100 Rhodamine DAM
Polyglutaminated-tubulin	1:100 in Saponin Buffer	1:100 Rhodamine DAM
M6PR	1:200 in Saponin Buffer	1:100 Rhodamine DAM
CD63	1:200 in Saponin Buffer	1:100 Rhodamine DAM

Table 5: Antibodies used in detection of defined proteins in western-blot or immunocyto/histochemistry

3.2.10 Two-dimensional electrophoresis (2D-PAGE)

Proteins were isolated from RLN2 and EGFP stable transfected cells using 2D lysis buffer, purified with the trichloroacetic acid (TCA) based 2-D Clean-Up kit and measured with 2-D Quant Kit, all according to the manufacturer's instructions.

From each protein sample, 30 µg or 200 µg proteins were aliquoted and mixed with 2-D rehydration solution to the final 450 µl amount. The first dimension – isoelectric focusing (IEF) – was performed using the Immobiline DryStrip gel pH 3-10. The whole 450 µl protein solution was loaded on 18 cm porcelain Strip Holder and DryStrip gel was positioned. To avoid evaporation, the gel, placed in Strip Holder filled with proteins solution, was covered with Immobiline DryStrip Cover Fluid. Before high voltage 8 h IEF in IPGphor II IEF System, the gels were rehydrated for 12 h at 30 V. After 23 h of rehydration-IEF process, the gels were equilibrated in iodoacetamide (IAA) and dithiothretiol (DTT) diluted in 75 mM Tris-HCl buffer pH 8.8 for 15 min each. Thereafter, strips were connected to the 12.5% polyacrylamide gels by employing 0.5% agarose. Gel electrophoresis was performed using 2D anode-buffer in lower chamber and 2D cathode-buffer in upper chamber of Ettan Dalt-six-electrophoresis system. Gels were run overnight at 20°C, starting with 2.5 W per gel and after 30 min increasing to 5 W per gel.

The gels were fixed for 1 h in 10% acetic acid/ 40% EtOH solution at 4°C and washed three times for 20 min in 75% EtOH. Before 20 min staining in 5% silver nitrate, the solution was enriched with 250 µl 37% FA and the gels were sensitivated with 0.02% natriumthiosulfate for 1 min. To develop silver stained gels the developing solution containing 3% natrium carbonate was applied. After 5 min the reaction was stopped by incubation in 5% acidic acid solution for 10 min.

The gels with stained proteins were scanned by a flat bed scanner. For identifying and evaluating protein spots, Phoretix 2D analysis software was used. The advantage of this software is the creation of virtual gels consisting of spots created by measuring each spot of three repeated gels. Spots on the virtual gel are an average value of the corresponding proteins in repetitive gels.

3.2.11 MALDI-ToF mass spectrometry

Spots of interest were excised from gels, chopped into 1 mm^3 cubes and dried in a vacuum concentrator. Gel-spots were then destained with 100 mM potassium ferricyanide/30 mM sodium thiosulfate, washed with HPLC water, shrunk with acetonitrile (ACN) and dried in a vacuum concentrator. Proteins were reduced (100 mM dithiothreitol in 100 mM NH_4HCO_3), alkylated (55 mM iodoacetamide in 100 mM NH_4HCO_3) and rehydrated with 30-50 µl cold trypsin solution (15 µg/ml). Digestion was performed for 16-24 h at 37°C in digestion buffer containing 50 mM NH_4HCO_3 and 5 mM $CaCl_2$. Peptides were twice extracted with 50% ACN/5% TFA (Trifluoroacetic acid) and dried. Desalting was performed with ZipTip containing C18 reverse-phase medium. Eluted peptides were dissolved in 50% ACN/0.1% TFA, combined with a matrix (a-cyano-4-hydroxy-trans-cinnamic acid) in a 1:1 ratio and spotted onto the sample plate. Protein spots were analysed with Mass spectrometry Unit Voyager DePro. Mass spectra were calibrated with standard kit (Applied Biosystems) containing des-Arg^1-Bradykinin, Angiotensin I, Glu^1-Fibrinopeptide B, Neurotensin, b-Galactosidase and Glycogen Phosphorylase. Spectra were reconstructed with DataExplorer software and analysed employing Mascot DataBase. One possible missed cleavage for trypsin was allowed and mass tolerance was set to 100 ppm.

3.2.12 Stable transfection of FTC-133 and FTC-238 cells

The pIRES-EGFP (EGFP) vector as well as a plasmid with gene insert (RLN2-EGFP) (gift from Dr J. Silvertown, Division of Stem Cell and Developmental Biology, Ontario Cancer Institute, University Health Network, Toronto, Ontario, Canada) were transformed into the *Escherichia coli* cells and left to grow on semi-solid LB-A (Luria-Bertani containing ampicillin) medium overnight at 37°C. Created colonies were transferred into separate tubs with liquid LB-A medium and multiplied overnight at 37°C. The DNA plasmid from *E. coli* was isolated using DNA midi isolation kit, according to the manufacturer's instructions.

Two thyroid carcinoma cell lines FTC-133 and FTC-238 were chosen for stable transfection with pCMV-preproRLN2-IRES-EGFP or pCMV-IRES-EGFP vectors. Transfection was performed in 0.5 ml OPTIMEM medium in a six-well plate using

1 μg plasmid DNA and 5 μl Lipofectamine 2000 reagent. After 6 h the transfection medium was replaced with 10% FCS DMEM growth medium for 24 h. Stable transfected clones were selected after applying selection medium (800 μg geneticin/ml DMEM/F12 medium 10% FCS). Transfectants with the emission of green fluorescence were chosen for further investigation. Over-expression of relaxin 2 was also verified by RT-PCR and western blot. Three clones demonstrating overexpression of RLN2 were chosen for further analysis.

3.2.13 Enzyme-Linked Immunosorbent Assays (ELISA)

Two separate ELISA assays were performed to detect secreted relaxin 2. Transfectants overexpressing relaxin 2 and EGFP mock cells (negative control) were seeded at $5x10^4$ cells per well. Secreted relaxin 2 was determined according to manufacturers' instructions and measured using an ELISA reader at 450 nm.

3.2.14 cAMP assay

Intracellular cAMP was measured with cell clone 10 of relaxin 2 transfectants. $1x10^4$ cells were seeded in each well of a 96-well plate and cultured for 24 h. For 2 h cells were pre-incubated with 3-isobutyl-1-methyl-xanthine (IBMX) at 37°C as cAMP sensitizer in cells. Medium was replaced with 200 μl of fresh serum-free medium as control for forskolin and relaxin 2 treated cells, serum-free medium containing 10 μM forskolin, an inducer of cAMP accumulation used as a positive control for relaxin 2 treated cells, and relaxin 2, as well as supernatants (200 μl) of FTC-133-EGFP (negative control) and relaxin 2 clones. All supernatants were collected after overnight serum-free culturing of $8x10^4$ cells in a six-well plate and centrifuged at 3000 x g for 30 min to remove remaining cells. The whole cAMP assay was performed according to manufacturer's instructions and levels of cAMP were measured at 450 nm in microplate readers after adding 1 M H_2SO_4 to stop the reaction.

3.2.15 Small interfering (si) RNA

The day before transfection, FTC-133 and FTC-238 cells were seeded in a six-well plate at a concentration of $1x10^4$ cells per well. The next day cells were washed with PBS and serum-free DMEM/F12 medium and transfected. Non-silencing siRNA

(siNC) conjugated to Alexa Fluor 555, coding sequence not matching any known human gene (1027099: 5'-AATTCTCCGAACGTGTCACGT-3') served as a negative control. Specific genes were silenced using single RXFP1 siRNA (5'-CTGCAGTTACCTGCTTTGGAA-3') for sequences located in exon 15, in concentration 300 nM or a combination of two specific RXFP1 silencing sequences at a concentration of 50 nM each (5'-GCTCCAGACCTTGGCAAAGAC-3' in exon 4 and 5'-TACTAGATAGGAATTGAGTCTCGTTGATT-3' in exon 20).
Transfection was performed in serum-free OPTIMEM medium using Lipofectamine 2000 reagent. After 24 h transfection medium was replaced with DMEM/F12 medium. The strongest effect of RXFP1 silencing was seen after 72 h.

3.2.16 MTT assay

MTT assay was performed with 2500, 5000 and 10000 cells under serum-free conditions. For measuring vitality of the cells, MTT solution was added (20 µl/well). After 4 h of incubation at 37°C, supernatant was decanted and 100 µl DMSO was applied to each well. Absorbance was measured at 570 nm using Spectra Rainbow ELISA and analysed using easyWin screening ELISA program.

3.2.17 Colorimetric BrdU proliferation test

Colorimetric BrdU proliferation ELISA was used for all transfectants: RLN2 cl.4, cl.10, cl.11 and EGFP as control. The assay was performed for 2500, 5000 and 10000 cells according to manufacturers' instructions. Before 30 min of incubation with BrdU antiserum, cells were air-dried and blocked with 200 µl per well blocking reagent for 30 min at RT. Thereafter, cells were incubated with 100 µl/well substrate solution for 10 min at RT. To stop the reaction 25 µl 1 M H_2SO_4 was added to each well and incubated for 1 min at 300 rpm. Colorimetric reaction was measured within 5 min at 370 nm in ELISA reader.

3.2.18 Luminometric ATP assay

Cells stably overexpressing relaxin 2 as well as EGFP transfectants were seeded at concentrations of 2500, 5000 and 10000 cells per well and grown overnight in a humidified incubator. To each well, 100 µl substrate was added, incubated in a

shaker and on bench tops at RT for 2 and 10 min respectively. Luminescence was measured using a Sirius Luminometer.

3.2.19 Motility and migration assays

The motility and migration assays were performed in 24-well Transwell chambers. The upper and lower culture chambers were separated with polycarbonate 8 µm porous membrane. For migration assays a separating filter was covered with 50 µg/ml human elastin, 1 mg/ml gelatine or collagen. To investigate the effect of recombinant relaxin 2 on motility or migration, $1x10^4$ cells of FTC-133 or FTC-238, suspended in FCS-free medium, were seeded in the upper chamber and incubated for 24 h in the absence or presence of 100 ng/ml or 500 ng/ml relaxin 2 in the lower chamber.

To determine the autocrine effect of investigated proteins, $1x10^4$ stable transfectants of FTC-133 and FTC-238 expressing relaxin 2, as well as FTC-133-EGFP and FTC-238-EGFP controls, were seeded in the upper chamber and let migrate for 24 h. To determine paracrine effects, $1x10^5$ cells of FTC-133/238-EGFP or FTC-133/238-RLN2 transfectants were seeded in a 24-well plate, serving as a lower chamber of motility/migration module.

Control experiments included the incubation of FTC-133 and FTC-238 wild-type cells with several concentrations of recombinant relaxin 2 as well as heat inactivated proteins, incubation with dilutions of supernatants collected after 24 h culture of stable transfectants and incubation of wild-type cells after suppressing expression of RXFP1 by siRNA.

Migrated cells were washed with PBS, fixed for 10 min in ice-cold MetOH-PBS followed by 20 min in ice-cold MetOH and stained in 0.1% toluidine blue solution in sodium carbonate. Stained cells were counted by light microscopy in five separate fields per filter.

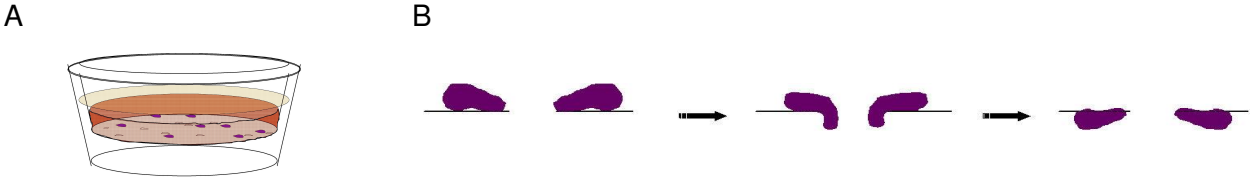


Figure 6: Migration assay chamber chared with 8 µm pored membrane (A). Cells are seeded in the upper chamber and let migrate to the lower chamber like shown in the schema (B).

3.2.20 Soft agar

The colony soft-agar assay was performed in a six-well plate. The bottom layer consisted of 5 ml of 3% agar in sterile water, 3 ml of FCS, 45 µl of geneticin (50 mg/ml), 300 µl of a 1:1000 dilution of amphotericin B (stock 250 µg/ml) and 900 µl of 1:1000 dilution of gentamicin (stock 10 mg/ml), added to 30 ml DMEM/F12 medium. This agar was portioned 1.5 ml per well and solidified for approximately 10 min at RT. In this time the upper layer was prepared from 1.6 ml of 3% agar, 10% FCS, 22.5 µl geneticin (50 mg/ml), 150 µl (1:1000 dilution) amphotericin B (250 µg/ml), 450 µl (1:1000 dilution) gentamicin (10 mg/ml) in 15 ml medium. The upper layer was mixed and portioned at 1 ml per tube. To the tubes were separately added EGFP and RLN2 thyroid carcinoma cells at the defined concentrations (25000, 50000 or 100000) and left on the bench top for 15 min to solidify. Both layers were covered with 1 ml medium, which was changed every week. After 4-6 weeks the living cells colonies were stained overnight in the incubator with 200 µl of iodotetrazolium chloride. Coloured colonies were examined by light microscopy.

3.2.21 MMP-2/MMP-9 activity assay

The MMP-2/MMP-9 activity assay was used to measure the activity of matrix metalloproteinase 2 (MMP-2) according to the manufacturers' instructions. The defined number (20000, 50000 or 100000) of cells was seeded in the small flasks in 2 ml serum-free medium and let grow. After two days the supernatants were collected, centrifuged for 30 min at 2000 x g to remove dead cells and concentrated with centricone 30 tubes. Samples were mixed with substrate specific for MMP-2/MMP-9 and diluted in PBS to the end substrate concentration of 100 µM and then incubated at 37°C. The absorbance of product created as a result of MMP-2/MMP-9 activity was measured after 30 and 60 min of incubation.

3.2.22 Gelatin zymography

Total cell lysates for zymography were isolated using 1x not reduced protein isolation buffer (20 mM HEPES pH 7.4, 1% Triton X-100, 10% glycerol, 2 mM EGTA in H_2O) without addition of proteases inhibitor. Protein concentration was measured using the BCA method according to manufacturers' instructions. Proteins were resolved on a

gelatine-polyacrylamide-SDS gel (gelatine-SDS-PAGE; 33% acrylamide, 25% TRIS pH 8.8, 1% SDS, 1 mg gelatine, 3% APS, 0.04% TEMED in H_2O). Electrophoresis was performed at 20 mA/gel and after 1.5 h the SDS was washed out in SDS wash-out buffer (2.5% Triton X-100 in H_2O). The gels were then incubated overnight at 37°C in activating buffer (50 mM Tris/HCl pH 8.0; 200 mM NaCl, 10 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.02% NaN_3) and stained with Coomassie Staining Solution. Densitometric data were obtained using Kodak Digital science 1D software.

3.2.23 Xenotransplantation of stable transfected thyroid carcinoma cells

Two relaxin 2 transfectants (cl.4, and cl.10) and EGFP transfected cells were xenotransplanted into three to four week old NMI nude male mice. The FTC-133-EGFP, FTC-133-RLN2 cl.4 and FTC-133-RLN2 cl.10 were collected from culture flasks and injected subcutaneously in serum-free medium with 100 µl sodium pyruvate at two positions into the axillae of each animal in 0,5 ml aliquots containing $2x10^7$ cells. Tumour growth was determined every $2^{nd}/3^{rd}$ day by measuring the widest, narrowest and deepest part of the tumour. Protocols involving animal experiments underwent an ethical review process by the institutional animal care and use of the Medical Faculty of the Martin-Luther-University, Halle-Wittenberg.

5 mice with EGFP (total of 10 injection sites)
6 mice with RLN2 cl.4 (12 injection sites)
5 mice with RLN2 cl.10 (10 injection sites

3.2.24 Statistical analysis

Statistical analysis was carried out with SPSS 12.0 and Excel software, and all experimental parameters were calculated for statistical significance using ANOVA, Mann-Whitney and Student's t-test. P values of < 0.05 were considered to indicate statistical significance. Densitometric analysis was carried out with Kodak Digital Science 1D software.

4 Results

4.1 Relaxin 2 in human thyroid carcinoma

The mRNA expression of relaxin (RLN2) and the G-coupled receptor RXFP1 were examined in adenoma (n=10), goiter (n=10), Graves' (n=9), follicular thyroid carcinoma (FTC) (n=17), papillary thyroid carcinoma (PTC) (n=11) and undifferentiated thyroid carcinoma (UTC) (n=10) tissues. All samples, independent of tumour stage, sex, or age contained the transcript of the RXFP1 receptor. Expression of relaxin 2 was exclusively found in human papillary (80%), follicular (78%) and undifferentiated thyroid carcinoma (100%), but not in benign tissues (Tab.6).

	RLN2	RXFP1
Goiter	0%	100%
Graves'	0%	100%
Adenoma	10%	100%
FTC	78%	100%
PTC	80%	84%
UTC	100%	100%

Tabele 6: Expression of relaxin 2 (RLN2) and RXFP1 in goiter, adenoma, Graves' and follicular, papillary and undifferentiated thyroid carcinoma tissues.

Immunohistochemistry results confirmed the expression of relaxin 2 in thyroid carcinoma tissues and low or negative relaxin 2 levels in benign tissues. All positive tissues showed cytoplasmatic localisation of relaxin 2 (Fig.7).

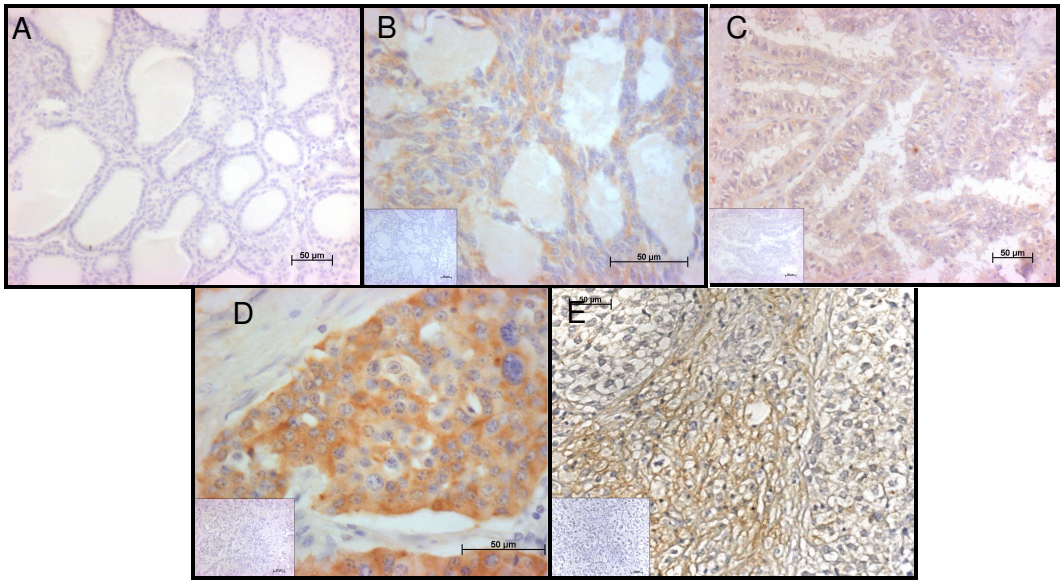


Figure 7: Immunolocalisation of RLN2 in human thyroid tissues. Human goiter tissue (A) shows no expression of RLN2. By contrast RLN2 positive immunostaining was detected in follicular (B), papillary (C) and undifferentiated (D, E) thyroid carcinoma tissues.

4.2 Expression of RLN2 and RXFP1 in human thyroid carcinoma cell lines

Semi-quantitative expression of relaxin 2 and the RXFP1 receptor were investigated in several thyroid carcinoma cell lines: follicular thyroid carcinoma FTC-133, FTC-236 and FTC-238, papillary thyroid carcinoma BC-PAP and undifferentiated thyroid carcinoma cell lines 8305C, 8505C, C-643 and HTh-74. All analysed cell lines revealed expression of relaxin 2 (RLN2) transcripts (Fig.8). These cell lines also expressed the RXFP1 receptor.

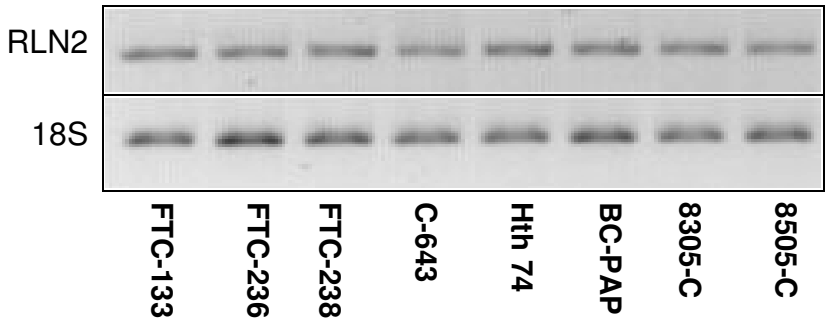


Figure 8: Expression of RLN2 in wild type thyroid carcinoma cell lines. RLN2 transcripts are expressed in follicular, papillary and anaplastic cell lines.

4.3 Characterisation of relaxin 2 (RLN2) expressing stable transfectants

Two follicular thyroid cell lines FTC-133 and FTC-238 were chosen for stable transfection with pCMV-preproRLN2-IRES-EGFP construct and pCMV-IRES-EGFP vector as a control. Expression of relaxin 2 transcripts in all transfectants was determined by RT-PCR (Fig.9A). Western blot analysis revealed a single immunoreactive band at approximately 18 kD, corresponding to proRLN2 (Fig.9B). Secreted relaxin 2 was measured using RLN2 ELISA. FTC-133-RLN2 cells released from 580 to 1050 pg/ml of RLN2, which is maximally 95-fold higher than the amount detected in EGFP controls (Fig.9C). Similar results were obtained for FTC-238-RLN2 transfectants, which secreted about 70-fold (730 pg/ml) more RLN2 compared with mock control (Fig.9C).

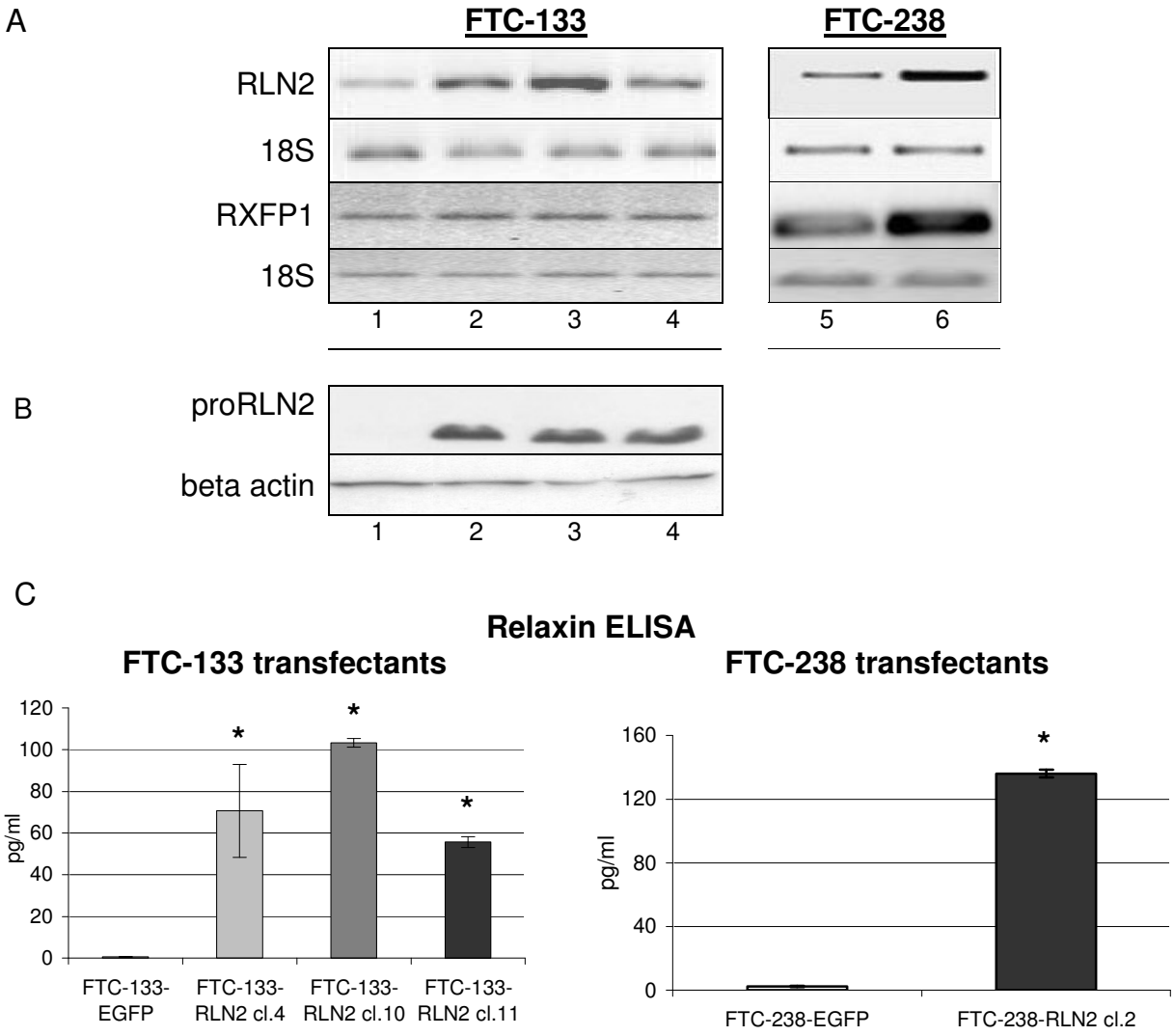


Figure 9: Relaxin 2 stable transfectants. FTC-133-RLN2 cl.4, FTC-133-RLN2 cl.10, FTC-133-RLN2 cl.11 display increased expression of relaxin 2 on mRNA level (A2, A3, A4 respectively) when compared to EGFP control (A1) as well as FTC-238-RLN2 cl.2 (A6) when compared to corresponding EGFP control (A5). All transfectants express RXFP1 receptor (A). Western blot analysis also revealed elevated expression of prorelaxin (proRLN2) in clones (RLN2 cl.4 – B2, RLN2 cl.10 – B3, RLN2 cl.11 – B4) when compared to MOCK control (FTC-133-EGFP- B1). Increased secretion of relaxin 2 was detected by ELISA (C).

To confirm bioactivity of secreted relaxin 2, the levels of cAMP in tested cells were measured. Forskolin treatment of FTC-133-RLN2 and FTC-133-EGFP cells responded with increased cAMP levels demonstrating a functional adenylyl cyclase system (Fig.10A). FTC-133-RLN2 cl.10, treated with the supernatants of three relaxin 2 clones cl.4, cl.10 and cl.11 and collected after 24 h culture, revealed weak but significant increases of cAMP. The highest influence showed the supernatant of FTC-133-RLN2 cl.10 (Fig.10A), which secreted the highest amount of RLN2 of all three clones. Similar results were obtained using wild-type (WT) cells of FTC-133 and FTC-238, where incubation with RLN2 increased levels of cAMP. Silencing of RXFP1

expression resulted with no cAMP elevation after incubation with recombinant relaxin 2 or the FTC-133-RLN2 cl.10 supernatant (Fig.10B).

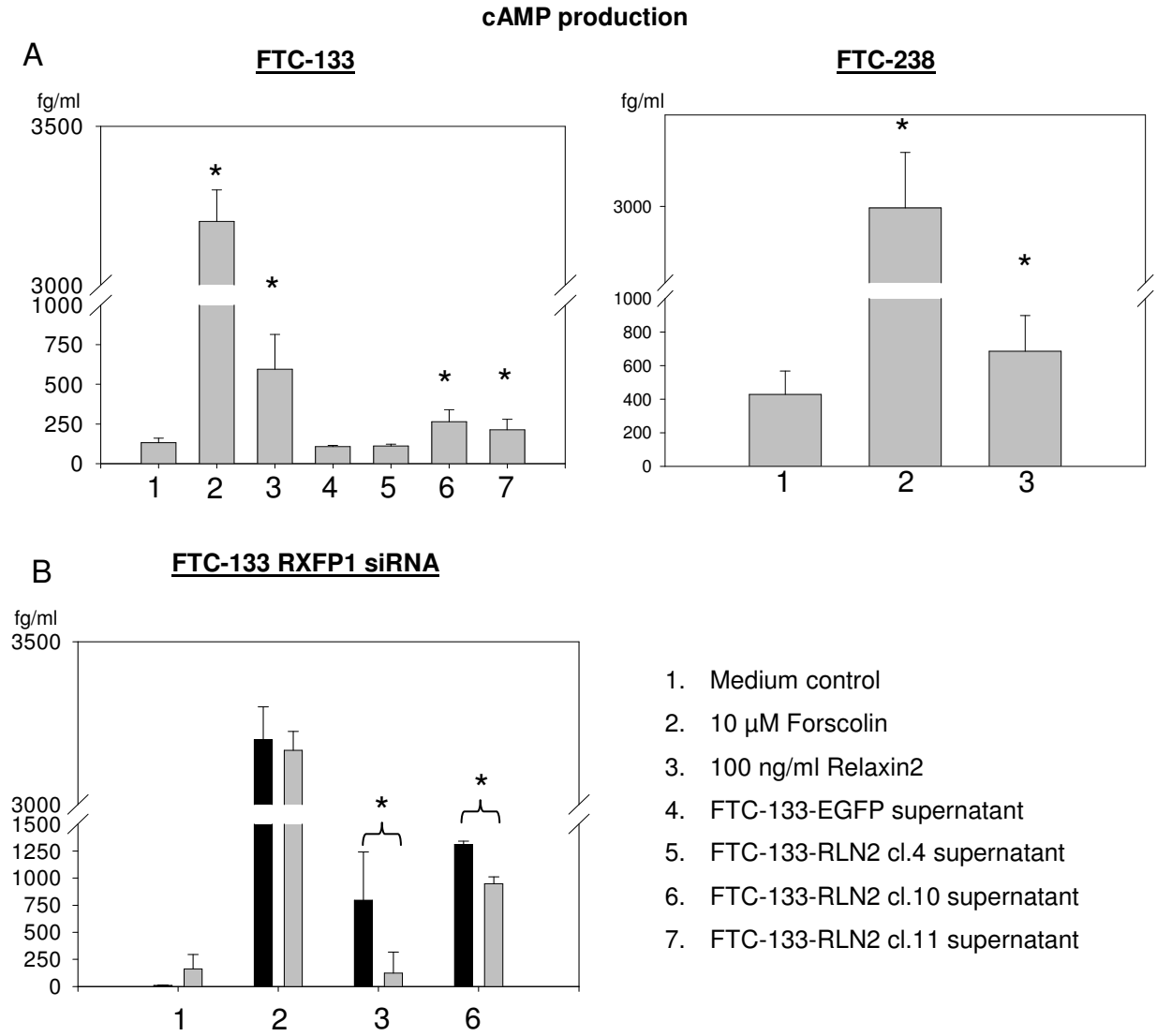


Figure 10: Representative cAMP immunoassays with responding FTC-133-RLN2 cl.10 transfectant incubated for 1 h with supernatants derived from RLN2 cl.4, cl.10, cl.11 and EGFP clone, which served as control (A), and FTC-238 wt cells incubated with RLN2 (B). Silencing of RXFP1 employing siRNA reduced cAMP response in cells (gray) when compared to controls (black) (C).

Relaxin 2 transfected cells displayed enhanced metabolic and mitochondrial activity measured by the formation of $NADH_2$-dependent formosan salt in MTT assay and increased intracellular ATP levels. After employing the RXFP1 receptor targeting siRNA, RLN2 transfected cells revealed no change in optical densities when compared with the corresponding control. Similar results were obtained with non-radioactive BrdU assays, which also showed no effect of relaxin 2 on the proliferation rate. FTC-238 cells did not show any alterations in MTT, ATP or BrdU assays.

4.3.1 Effect of relaxin 2 on motility of thyroid carcinoma cells

The potential influence of relaxin 2 on the invasive ability of thyroid carcinoma cells was investigated. Treatment with human recombinant relaxin 2 increased the motility of FTC-133 and FTC-238 by ca. 1.63 times and 5.9 times, respectively. Specificity of the relaxin-2-induced motility was verified by employing small interfering RNA (siRNA) or antisense (AS) techniques against the RXFP1. A single specific RXFP1-siRNA construct decreased RXFP1 transcript expression by half and the combination of the two specific RXFP1 antisense primers by 30%, as demonstrated by RT-PCR. In experiments performed on cells with silenced RXFP1, incubation with relaxin 2 did not enhance the motility of treated cells compared with controls (Fig.11A, B). Moreover, heat-inactivated RLN2 was unable to induce motility of thyroid carcinoma cells (Fig.11D). Employing different dilutions of supernatants derived from FTC-133-RLN2 cl.10 demonstrated the concentration-dependent effect on cell motility (Fig.11D). Incubation of the wild-type FTC-133 cell line with EGF, which served as a positive control, increased motility 1.59 times.

Relaxin 2 expressing clones migrated ca. 3.6 times faster through the filters than EGFP clones, confirming the autocrine effect of produced relaxin 2 (Fig.11E). Testing in a paracrine manner, the supernatant of transfectants enhanced motility of EGFP clones3.02 and RLN2 clone10 1.38 times, showing the combined auto- and paracrine effect (Fig.11F).

4.3.2 Cytoskeletal changes in thyroid carcinoma cells exposed to RLN2

Immunostaining of cytoskeletal proteins revealed the influence of relaxin 2 on the morphology of thyroid carcinoma cells. Incubation with 100 ng/ml of RLN2 induced visible, after F- or G-actin staining, the elongation of FTC-133 WT cells, previously observed for relaxin 2 stable transfectants (Fig.12).

Analysis of F-actin localisation in relaxin 2 transfectants (Fig.11B, E) revealed augmented polymerisation of actin, when compared to the mock cells (Fig.11A, F). Globular, non-polymerised actin (G-actin) was detected in all cell bodies, showing no differences between the transfectants.

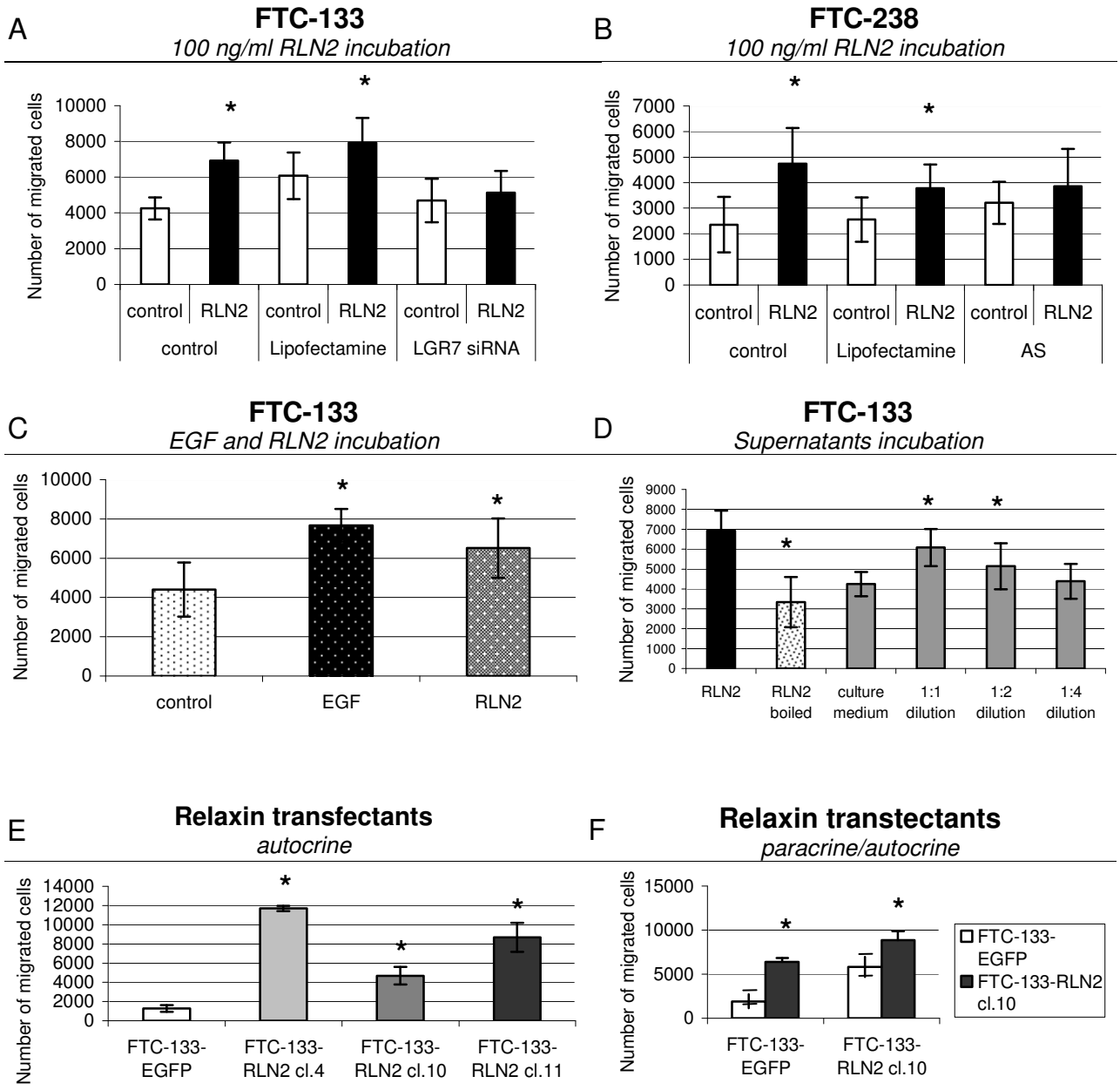


Figure 11: Influence of relaxin 2 on motility of follicular thyroid carcinoma cells. Incubation with 100 ng/ml relaxin 2 increases motility of FTC-133 (A) and FTC-238 (B) cells. Previous silencing of RXFP1 receptor reduces influence of relaxin 2 on the cells (A, B). Treating cells with 10 ng/ml EGF served as the control (C). Dilutions of supernatants derived from representative FTC-133-RLN2 cl.10 also elevated migration of FTC-133 cells in paracrine manner (D). In autocrine test relaxin 2 transfectants migrated faster than EGFP clone (E). Treating FTC-133-EGFP (paracrine) and FTC-133-RLN2 (paracrine) cl.10 with supernatants derived from FTC-133-RLN2 cl.10 cells confirmed influence of relaxin 2 on motility of transfected cells (F).

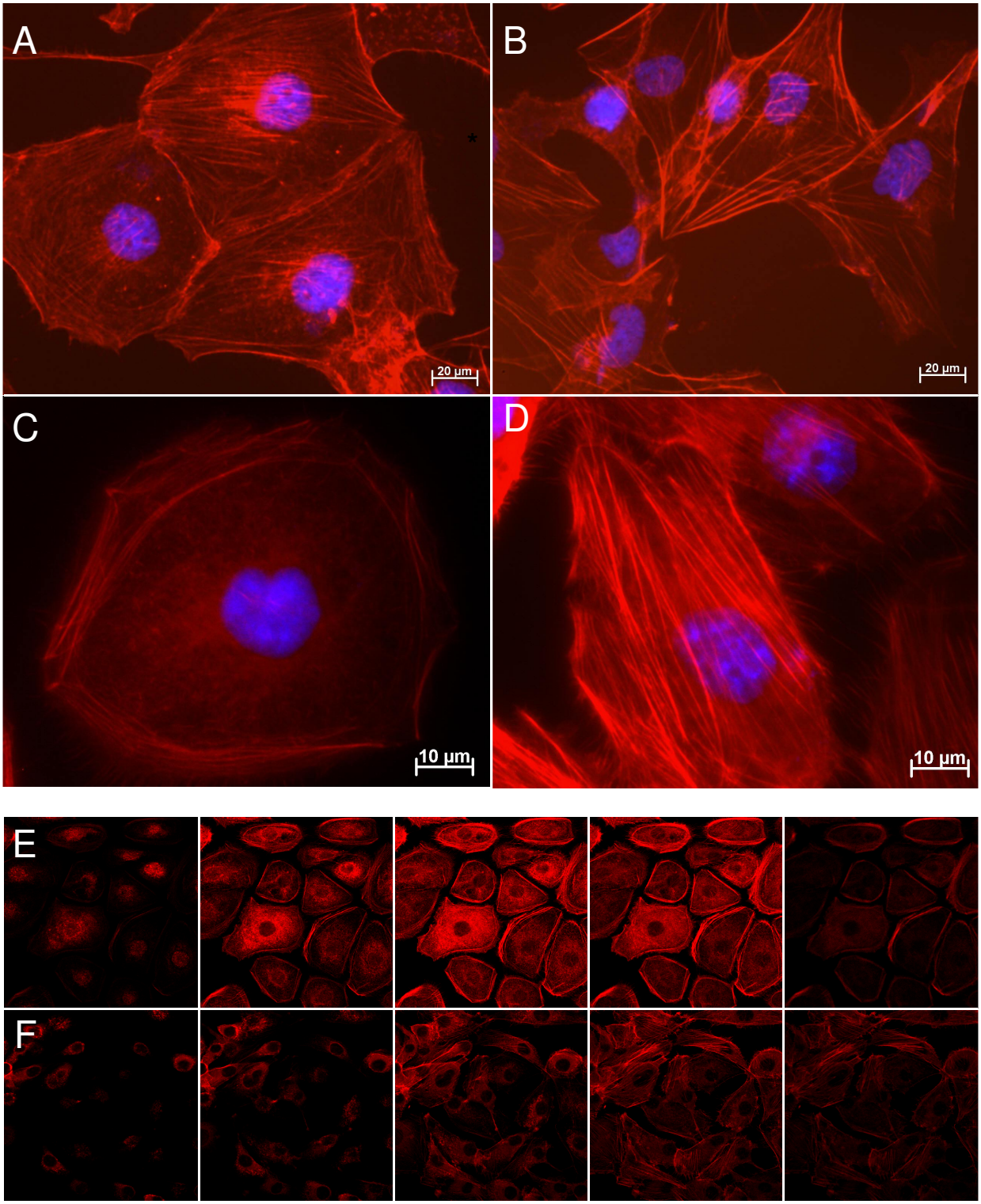


Figure 12: F-actin localisation in thyroid carcinoma cells. Immunostaining of Phalloidin in FTC-133 WT cells (A) and FTC-133 cells treated with RLN2 (B), as well as in mock (C) and relaxin 2 clones (D) revealed alterations in F-actin localisation in relaxin 2 influenced cells observed under light (A-D) and confocal (E, F) microscopy. The actin staining confirmed the morphological differences after relaxin 2 incubations in FTC-133 cells.

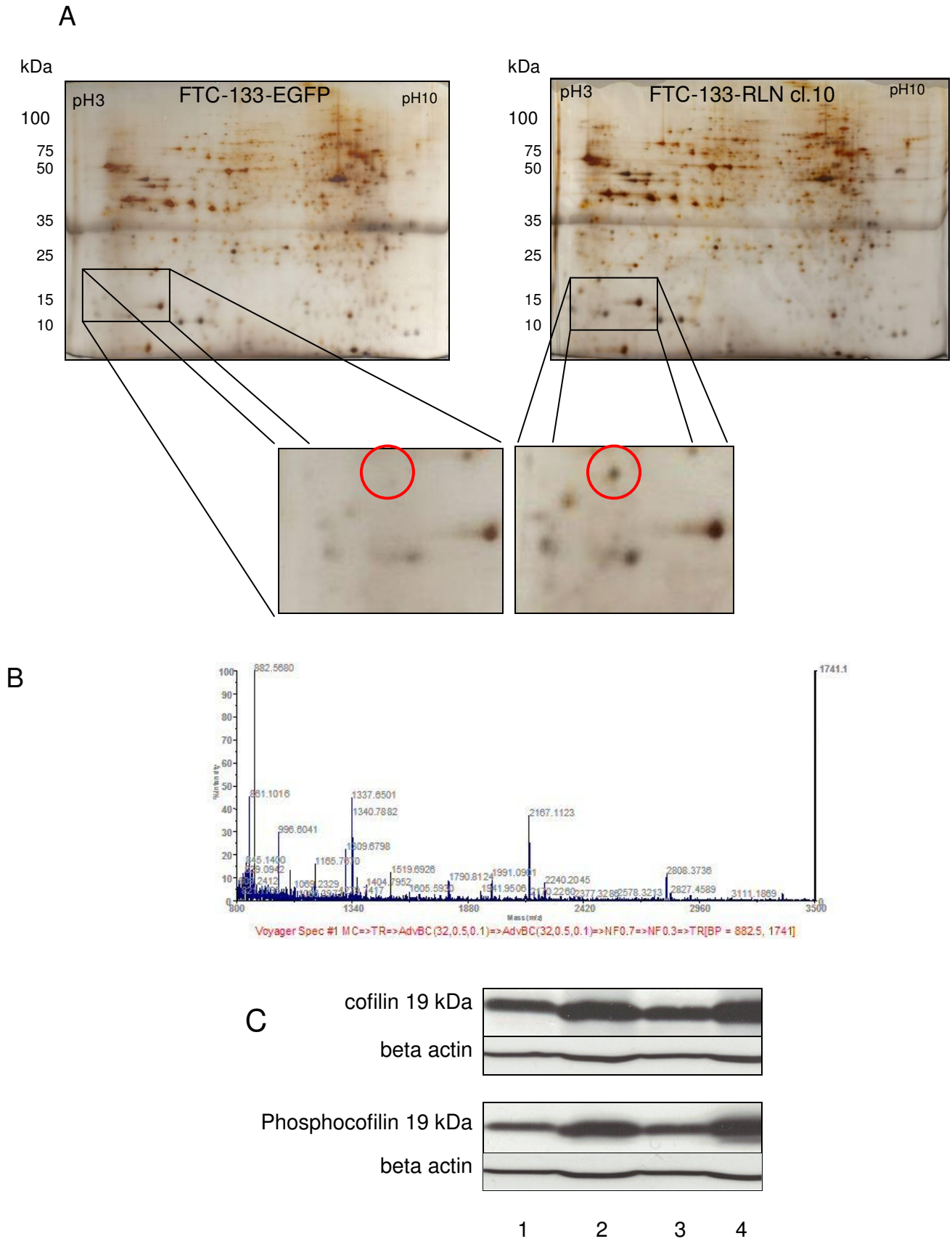


Figure 13: Expression of cytoskeletal proteins. The red marked spot on two dimensional electrophoresis gels (2D PAGE) of mock and FTC-133-RLN2 cl.10 cells, up-regulated in relaxin 2 clone (A), was identified, employing mass spectrometry analysis (MS) as ADF/cofilin1 (B). Westernblot analyses of all relaxin 2 transfectants (FTC-133-RLN2 cl.4- C1, cl.10- C2, cl.11- C3) revealed elevated expression of cofilin in both forms when compared with the mock control (C 4).

Silver stained two-dimensional electrophoresis gels (2D-PAGE) of representative relaxin 2 and mock clones (30 µg proteins each) displayed changes in general protein patterns consisting of 368 or 363 spots for FTC-133-EGFP and FTC-133-RLN2 cl.10, respectively. Mass spectrometry analysis performed on spots of molecular range between 15 and 30 kDa identified one of the proteins as ADF/cofilin1 (Actin depolymerisation factor). Its expression was upregulated in relaxin 2 transfectants (Fig.13A). Western blot analyses employing specific cofilin1 and phosphocofilin1 antibodies confirmed the increased expression of both phosphorylated and not phosphorylated forms of the protein in all three FTC-133-RLN2 transfectants compared with the mock control (Fig.13C).

4.3.3 Elastinolytic activity of transfectants.

Protein expression of cathepsin family, determined by westernblot analyses, revealed regulation of Cathepsin D and L. The other tested proteins, Cathepsins V, K, B, and H, were not influenced by expression of relaxin 2. Westernblot analyses revealed increased production of proCathepsin D (52 kDa) in relaxin 2 transfectants comparing to the control. Matured form was slightly decreased in relaxin 2 transfectants of FTC-133 cells and not detected in FTC-238 (Fig.14). Secreted protein did not show any significant differences.

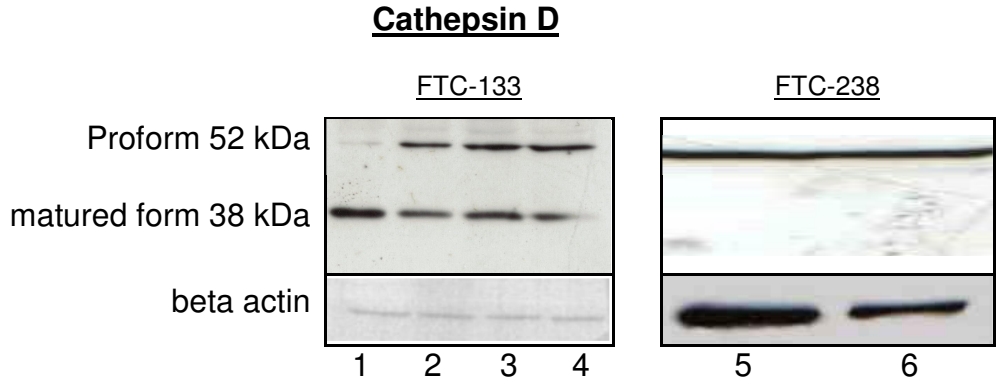


Figure 14: Production and processing of Cathepsin D in relaxin 2 transfectants. All relaxin 2 over-expressing cells of FTC-133 (2-4) and FTC-238 (5,6) cell lines revealed increased production of proCathepsin D when compared to EGFP transfected FTC-133 (1) or FTC-238 (5) cells. The matured form in FTC-238 cells was not detected.

The second regulated protein of this family was Cathepsin L. In the case of this protein, synthesis of proform (42 kDa) and its processing to the heavy (31 kDa) and

single chain-active form (24 kDa) were also increased in all relaxin 2 transfected cells compared with the mock control. Analysis of serum-free supernatants revealed the increased secretion of all forms from relaxin 2 clones (Fig.15).

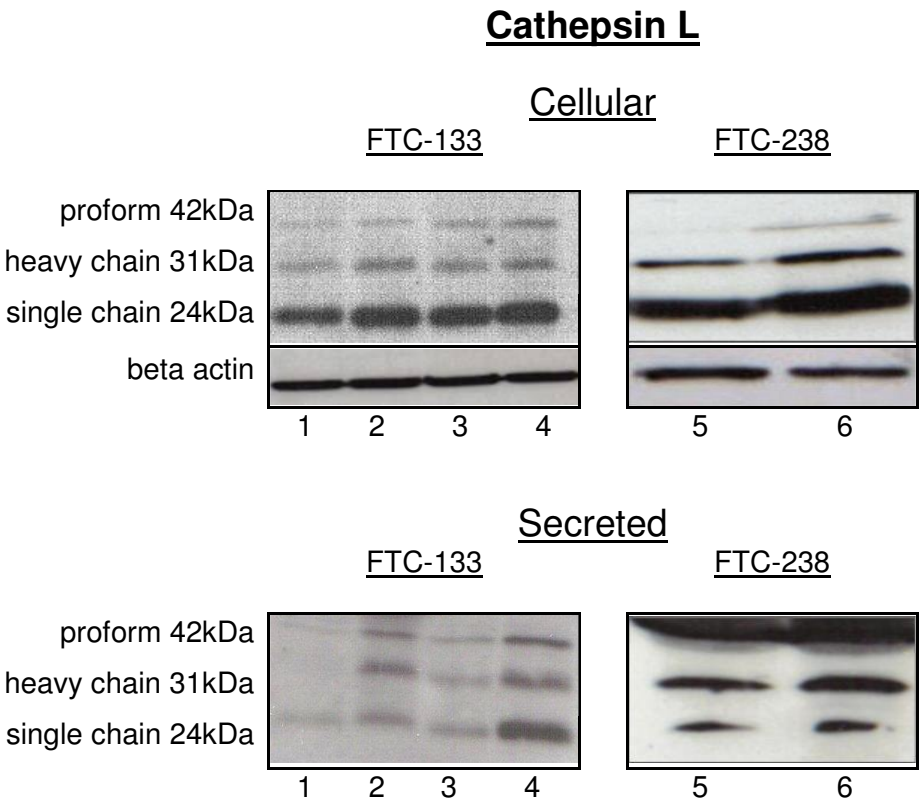


Figure 15: Increased expression and secretion of Cathepsin L. Relaxin 2 (A2-4, 6) over-expressing cells of FTC-133 (A1-4) and FTC-238 (A6) display increased expression of proform of Cathepsin L when compared to mock cells (A1, A5). Processing of proform to the matured forms of single and heavy chain was also faster in relaxin 2 transfectants than in EGFP cells (A). Serum-free supernatants of all clones of FTC-133 (B1-4) as well as FTC-238 (B5, 6) revealed increased secretion of proCathepsin L, as well as higher amount of both active forms - single and heavy chains, when compared to EGFP cells (B1, B5).

Immunocytochemistry revealed polar and nuclear localisation of (pro-) Cathepsin L (proform - D2G mAb and all forms - 33/1 mAb) in relaxin 2 clones of both FTC-133 (Fig.16) and FTC-238 transfectants compared with corresponding controls or wild-type cells. Confocal laser-scanning microscopy confirmed perinuclear or cytosolic localisation of Cathepsin L forms and revealed the presence of nuclear Cathepsin L in both FTC-133 and FTC-238 cells (Fig.16). No differences in the distribution of Cathepsin D were detected with a polyclonal anti-cathD antibody (Fig.17). Localisation of cathepsins receptor in Golgi apparatus – mannose-6-phosphate receptor (M6PR) –displayed perinuclear polar distribution, similar to Cathepsin L (Fig.17). By contrast, localisation of lysosomal marker – CD63

or other proteins connected with vesicular transport such as delta- and gamma-adaptins – was unaffected by the relaxin 2 expression.

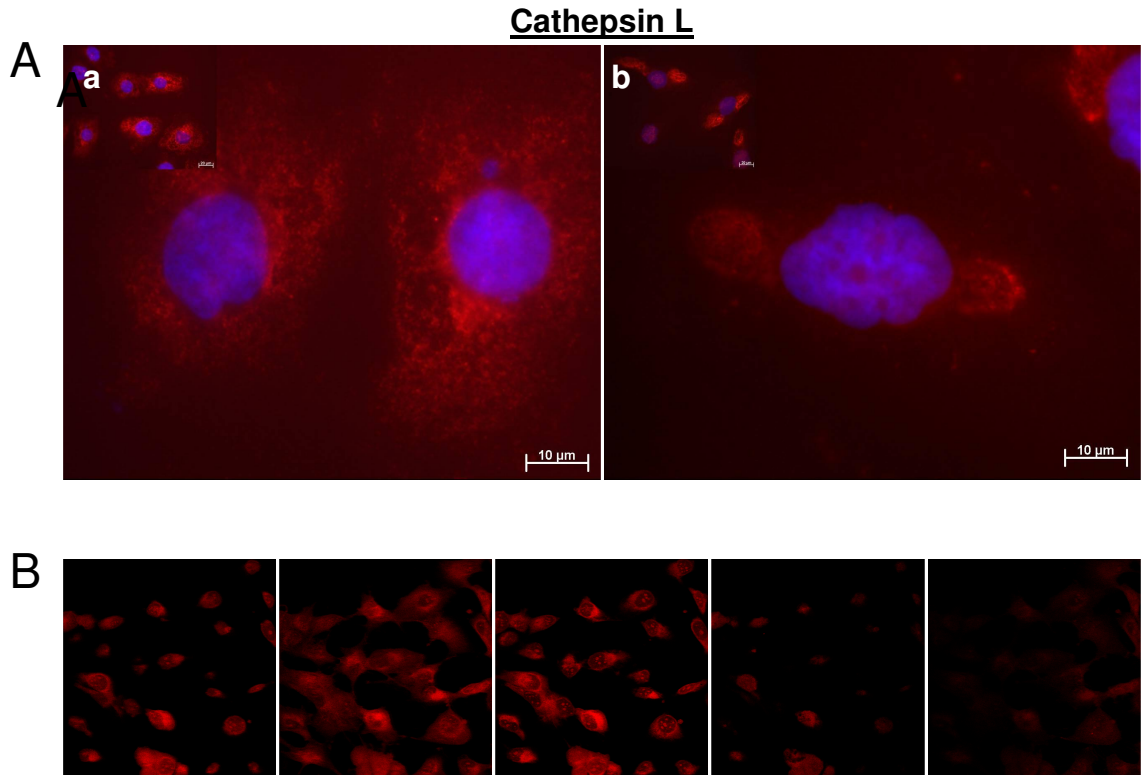
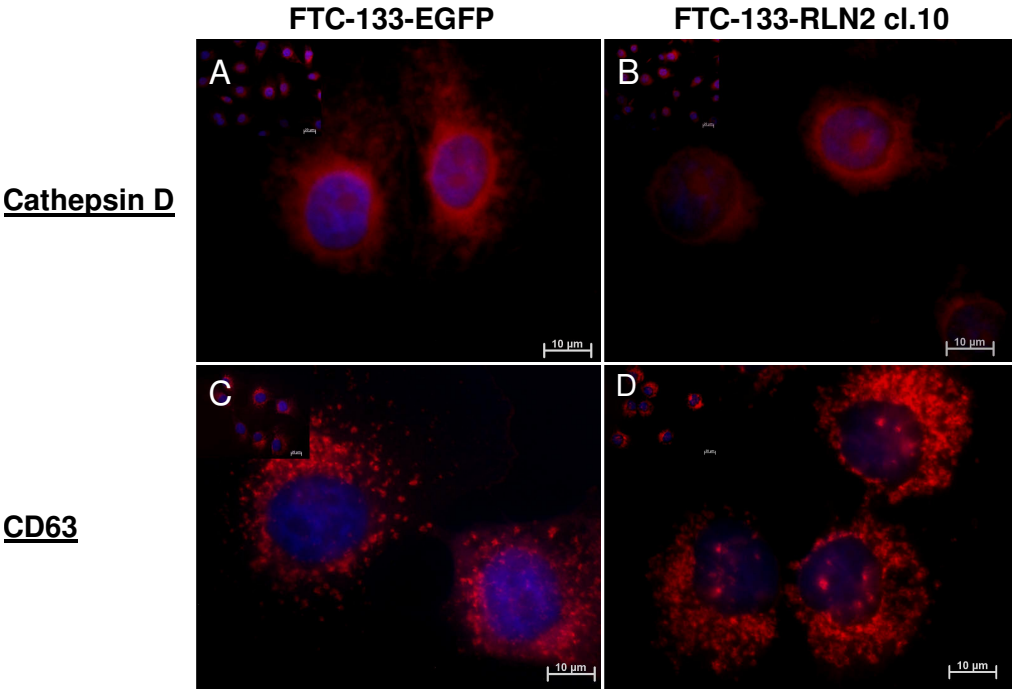


Figure 16: Polar localisation of Cathepsin L. Immunocytochemical analysis of Cathepsin L under light (A) and confocal (B) microscopy revealed polar localisation of this protein in relaxin 2 transfectants (A, B) when compared to MOCK control (A)



M6PR

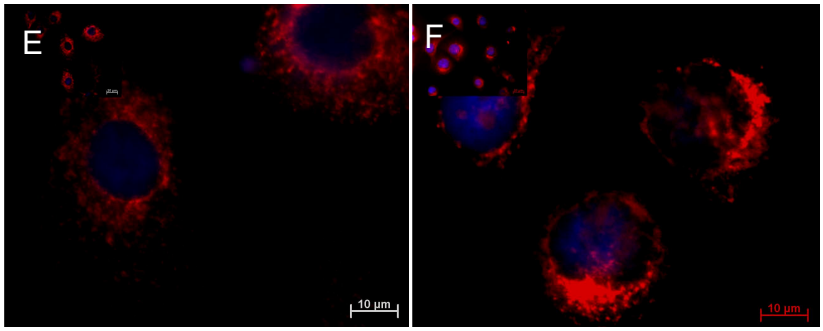


Figure 17: Cellular localisation of lysosomal markers Cathepsin D and CD63, and cathepsins receptor mannose-6-phosphate (M6PR). Localisation of both lysosomal markers: Cathepsin D (A, B) and CD63 (C, D) did not differ between relaxin 2 (B, D) transfected cells and MOCK (A, B). Localisation of M6PR – cathepsins receptor in relaxin 2 transfectants (F) displayed perinuclear, polar localisation, when in EGFP (E) transfected cells this protein is distributed cytosolic.

Previously observed increased expression and secretion of Cathepsin L suggest increased elastinolytic activity of relaxin 2 transfected cells. Hence we focused our further investigations on the ability of the cells to penetrate elastin matrix.

All relaxin 2 transfectants migrated faster through elastin covered filters than the corresponding controls. The FTC-133-RLN2 clones revealed 3.4 (cl.4), 3.9 (cl.10) and 5.1 (cl.11) times increases compared with mock cells, and the FTC-238-RLN2 clones 2.2 times. The FTC-238-WT cells treated with 100 ng of human relaxin 2 migrated 1.9 times faster than the medium control (Fig.18).

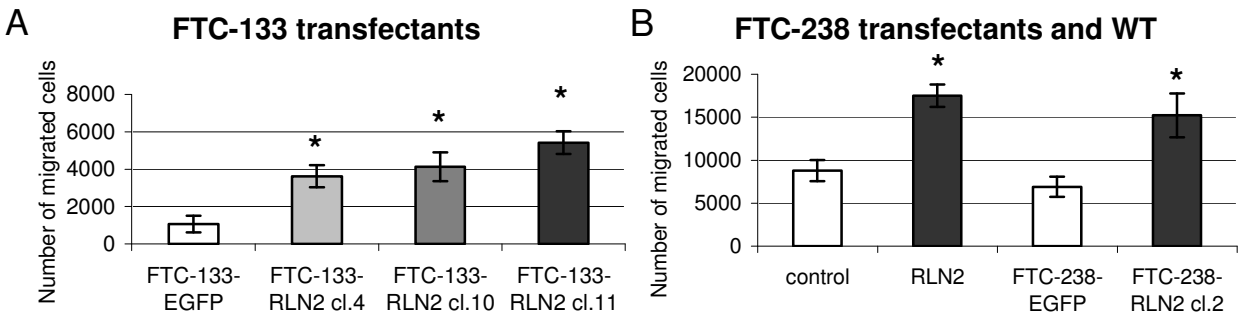


Figure 18: Elastin migration. Relaxin 2 expression induced elastinolytic potential of the follicular thyroid carcinoma cells FTC-133 (A) and FTC-238 (B). Additionaly incubation of FTC-238 cells with human recombinant relaxin 2 confirmed its enhancing effect on elastinolytic ability of FTC-238

4.3.4 Gelatinolytic/collagenolytic activity of relaxin 2 transfectants

Analysis of gene expression in transfectants revealed differences in expression of several gelatinolytic/collagenolytic enzymes and their inhibitors compared with controls. All examined cells expressed MMP-2 and MMP-13 (matrix-metalloproteases 2 and 13), MT1-MMP (membrane-bound matrix-metalloprotease 1),

ADAM23 (a disintegrin and metalloprotease 23), ADAMTS-1 and ADAMTS-5 (a disintegrin and metalloproteases with thrombospondin motifs 1 and 5) as well as all four TIMPs 1-4 (tissue inhibitors of metalloproteases 1-4).

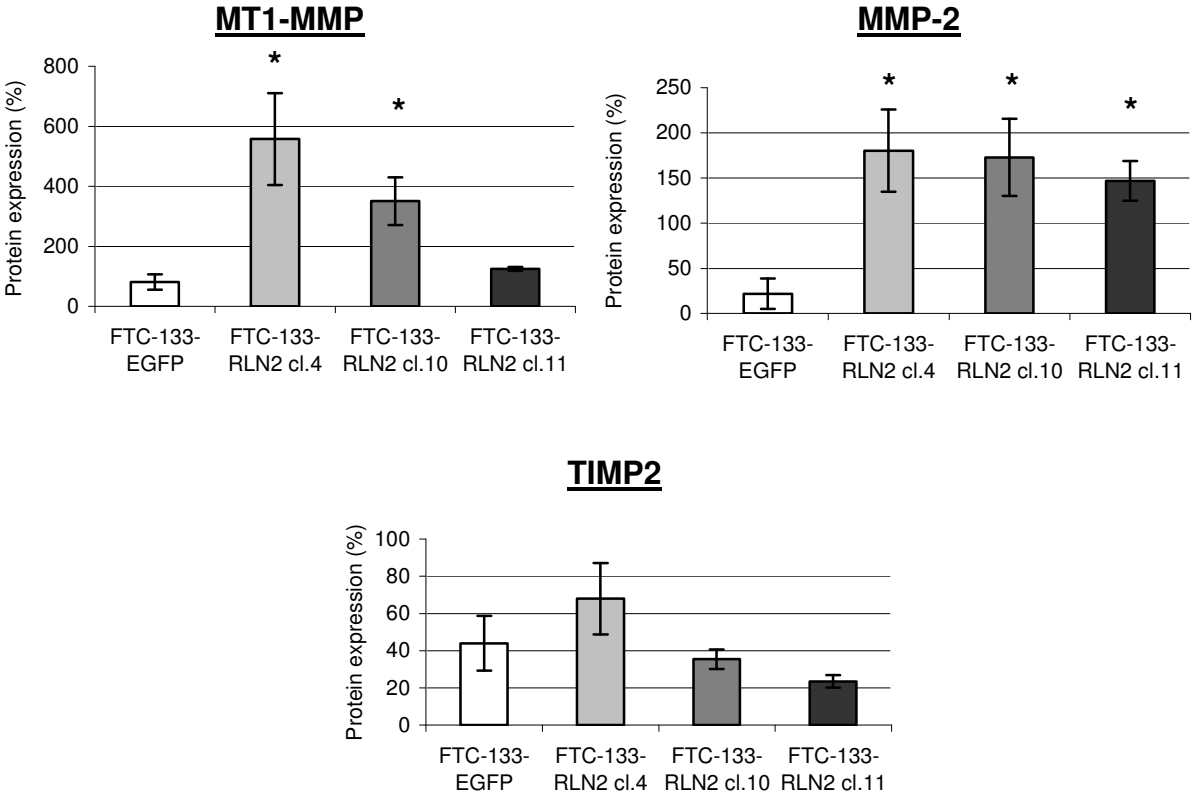


Figure 19: Expression of extracellular matrix affecting proteins – MT1-MMP/ MMP-2/TIMP2 complex. In two of three Relaxin transfectants expression of MT1-MMP collagenase is increased, in all relaxin clones MMP-2 expression was increased and TIMP2 was unchanged.

Western blot analysis revealed the increased expression of MMP-2 (gelatinase and collagenase) in all relaxin 2 transfectants, MT1-MMP in two of three relaxin 2 clones (RLN2, cl.4 and cl.10) (collagenase and MMP-2 activator) and no regulation of TIMP2 (an MMP-2 activation mediator and inhibitor) (Fig.19). Further investigations of metalloproteases expression revealed the presence of ADAMTS-1, increased expression of ADAM23 and ADAMTS-5 as well as down-regulation of the active form of TIMP3 (a potential inhibitor of ADAMs) (Fig.20) and elevated expression of MMP-13. These findings suggest an enhanced gelatinolytic/collagenolytic activity of relaxin 2 clones. Other proteins belonging to the metalloproteinases family, such as MMP-1 (collagenase), MMP-8 (collagenase) and MMP-9 (gelatinase), were undetected.

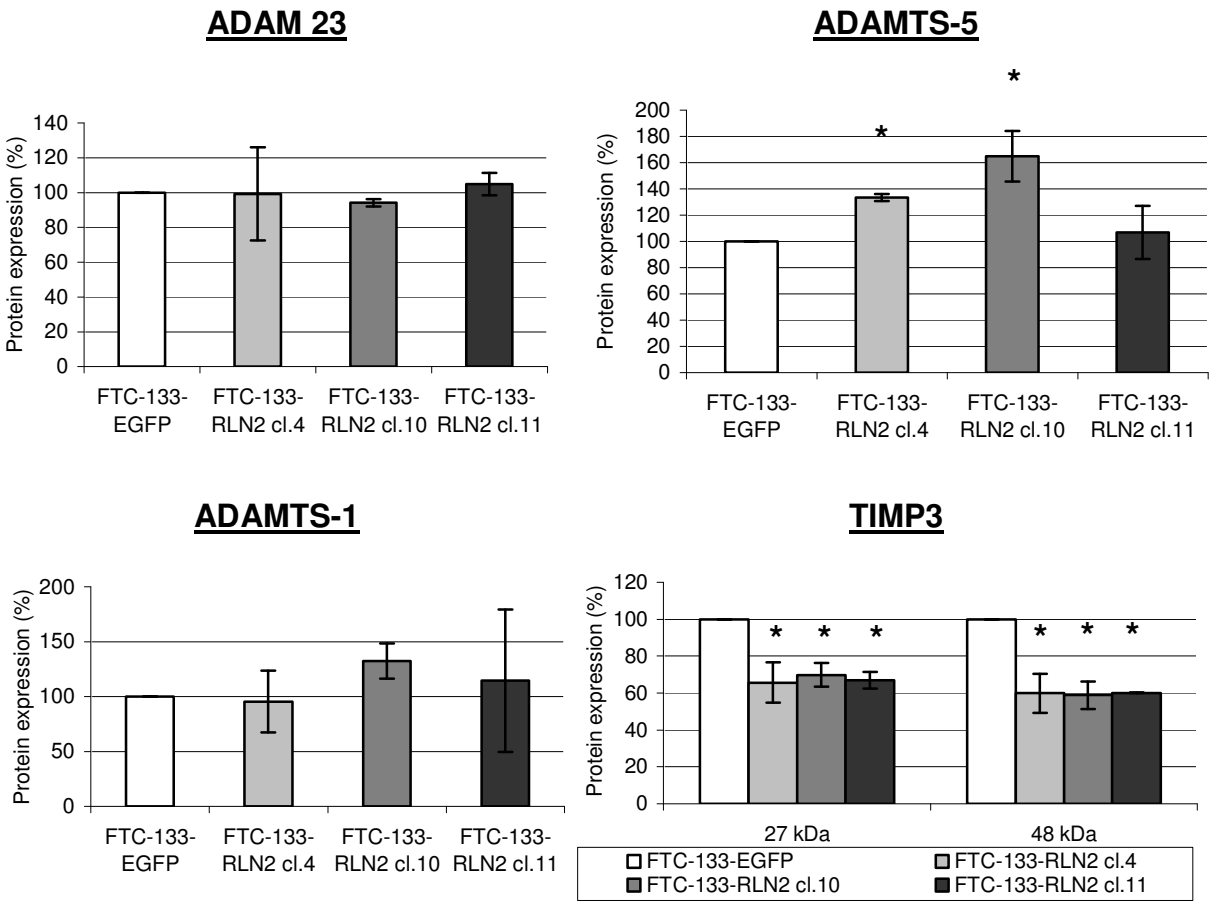


Figure 20: Expression of extracellular matrix affecting proteins – ADAMs. Expression of both ADAM23 and ADAMTS-5 is upregulated in FTC-133-RLN2 (2-4) clones, when ADAMTS-1 does not show any changes. Level of 50 kDa and 27 kDa forms of TIMP3 is decreased in all relaxin 2 transfectants when compared to the control.

4.3.5 Gelatine and collagen migration

All relaxin 2 transfectants investigated revealed an increased production of gelatinase/collagenase (MMP-2) or collagenases/gelatinases (MT1-MMP and MMP-13). Since those enzymes can digest ECM components, the penetrating activity of transfectants was tested. Filters were covered with gelatine or collagen (I/III in ratio 70%:30%). After 24 h migration through gelatine relaxin 2 transfectants did not differ from mock controls (Fig.21A), and wild-type FTC-133 cells treated with relaxin 2 revealed only slight but not significant increases in migration (Fig.21C).

Investigations on collagen matrix displayed high migration ability of all relaxin 2 clones compared with the FTC-133-EGFP control (Fig.21B). Incubation of wild-type cells with relaxin 2 displayed an increased migration rate of all cells compared with controls (Fig.21D).

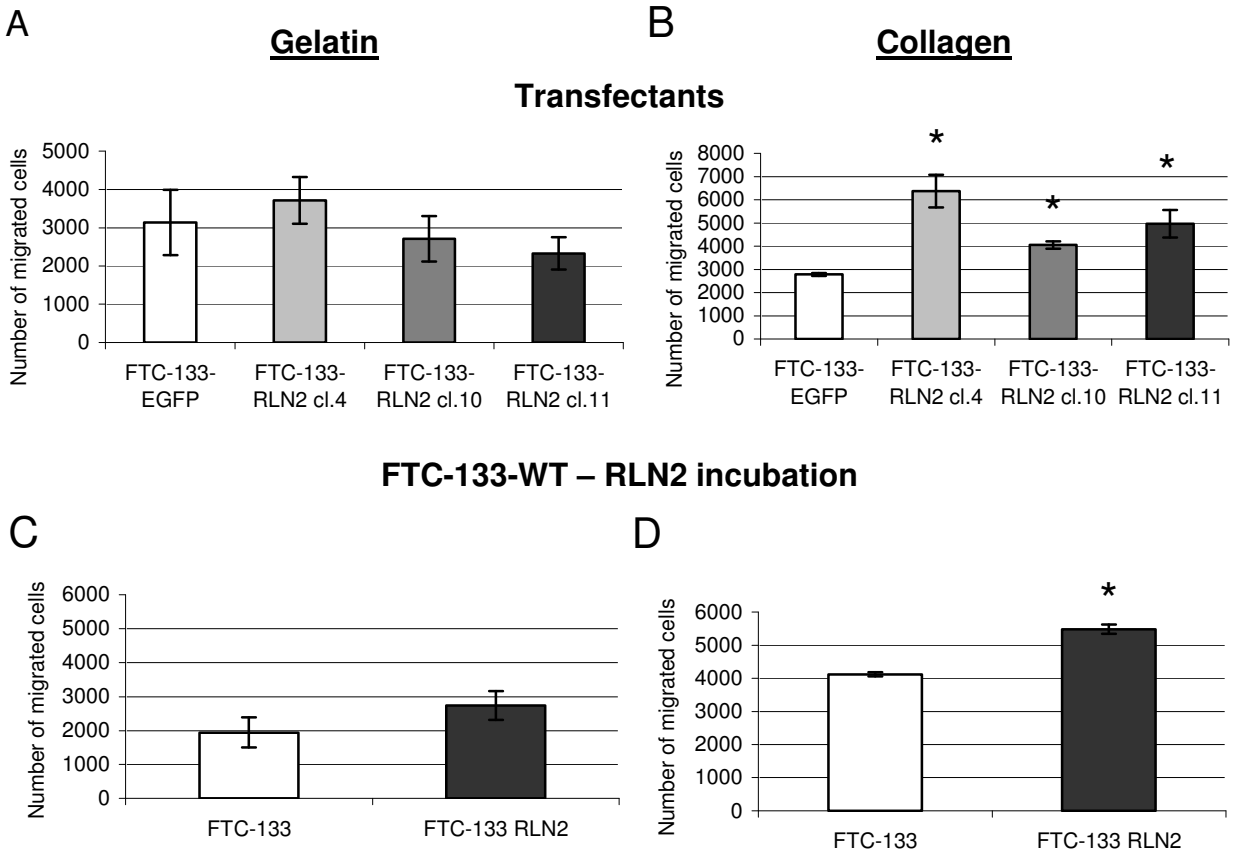


Figure 21: Gelatine and collagen migration. Relaxin 2 transfectants did not reveal any significant differences in migration through gelatine (A), however all clones migrated faster through collagen (B). Incubation of FTC-133 wild type cells with relaxin 2 did not increased gelatinolytic (C), but collagenolytic (D) activity of cells.

4.3.6 Activity of MMP-2

Since the western blot analysis revealed an increased production of MMP-2, a gelatinase and collagenase, and gelatine migration tests displayed no differences, further analysis of the activity of this protein were performed. Spectrophotometric analysis revealed no increased activity of MMP-2 in all relaxin 2 transfectants compared with the mock cells (Fig.22A). The treatment of wild-type FTC-133 with 500 ng/ml RLN2 did not influence this process either (Fig.22B).

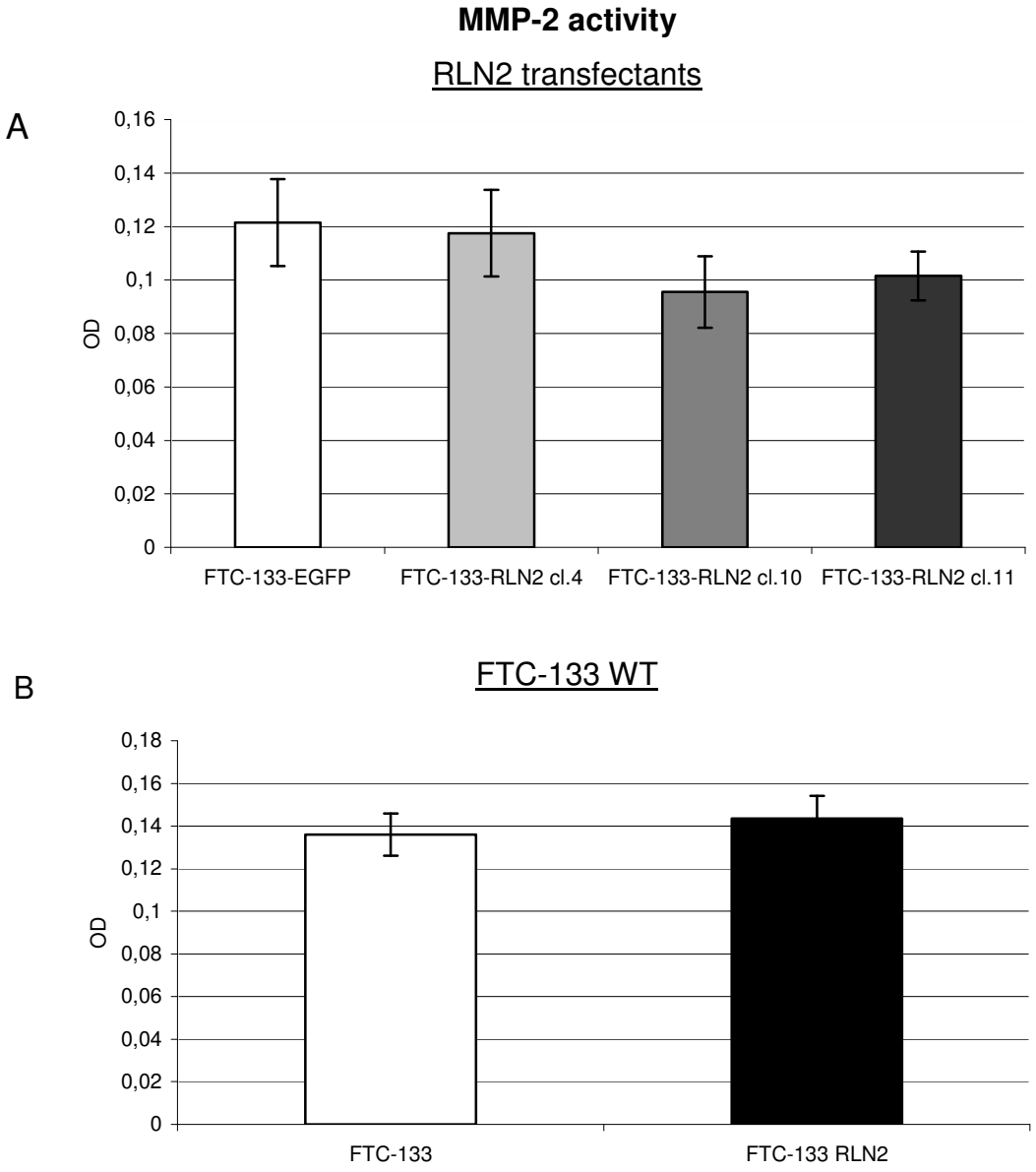


Figure 22: Spectrophotometrical analysis of MMP2 activity. Spectrophotometrical analysis of MMP2 activity revealed no increased activity in relaxin 2 transfectants compared with EGFP control (A). Incubation of wild type FTC-133 cell line with 500 ng/ml relaxin confirmed no influence of relaxin 2 on activity of MMP-2 (B).

To verify the gelatinolytic potential of the follicular thyroid carcinoma cells zymography was performed. All cells displayed the facility to digest gelatine; however incubation with relaxin 2 did not reveal any differences (Fig.23).

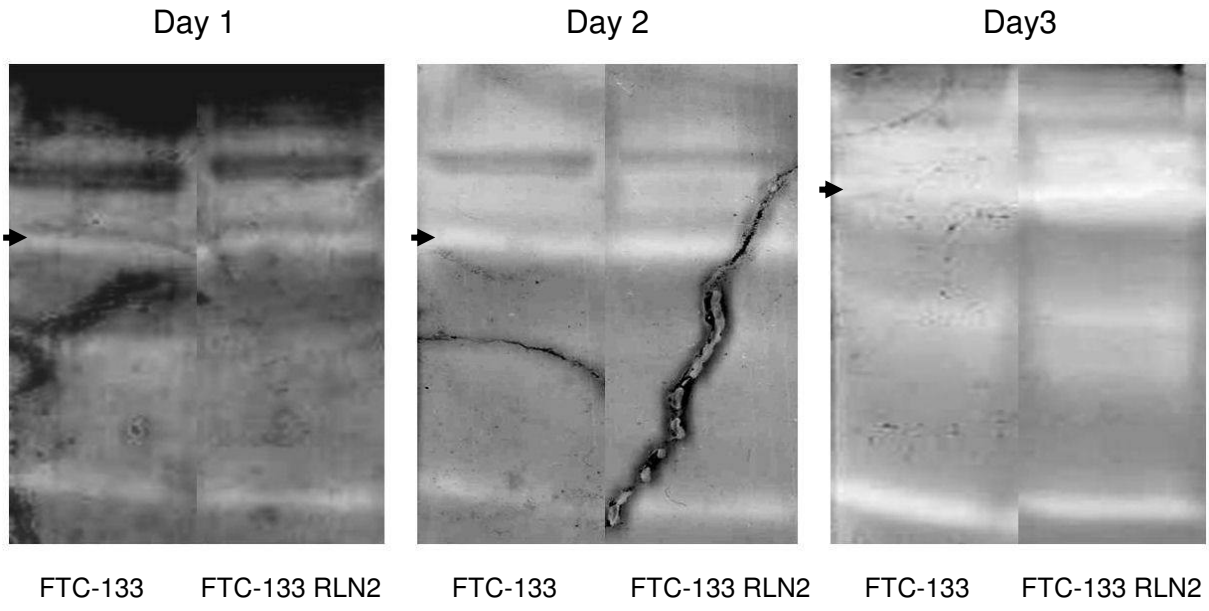
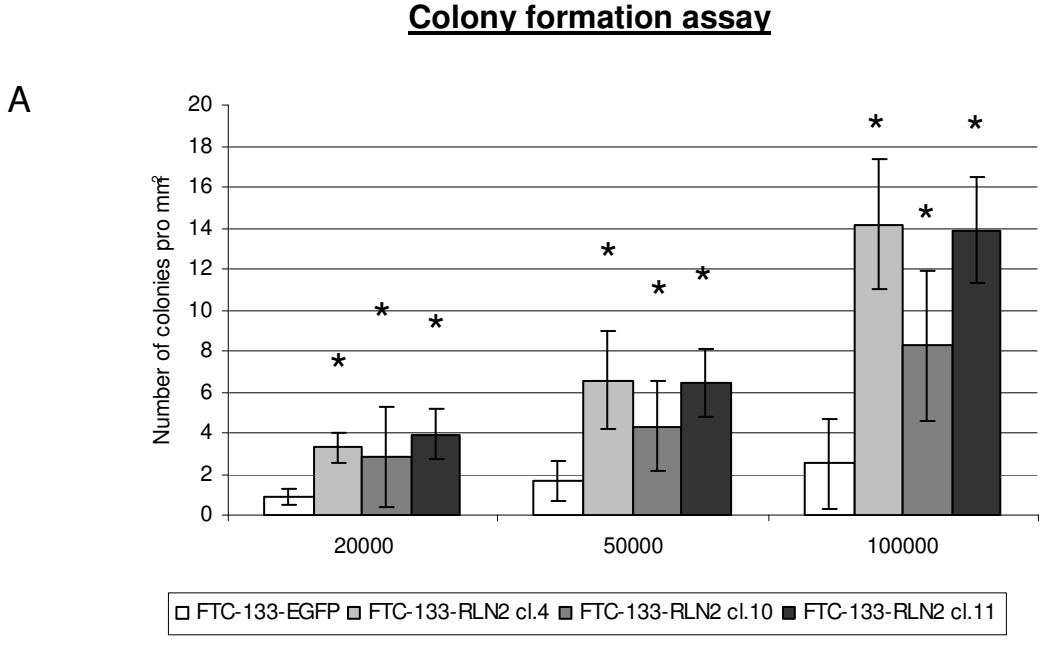


Figure 23: Gelatin zymography of FTC-133 cells treated with relaxin 2. Relaxin 2 treatment did not influenced gelatinolytic ability of FTC-133 cells.

4.3.7 Relaxin 2 in cell colony formation

The *in vitro* colony formation test, based on anchorage independent growth, describes the influence of relaxin 2 on the ability of the thyroid cells to create live colonies. All relaxin-2-exposed cells revealed, in all three concentrations of the cells, an increased ability to form colonies (2.5–5.5 times more than controls for cl.10 and cl.4 respectively) (Fig.24).



B

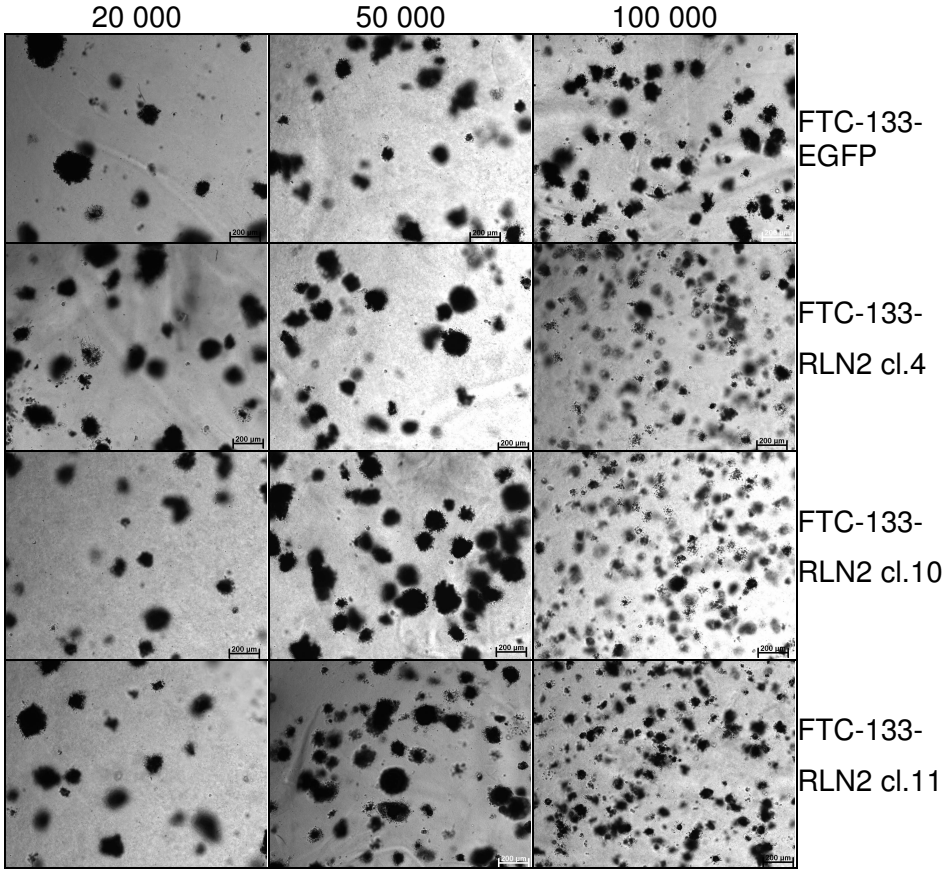


Figure 24: Soft Agar assay for colony formation. After six weeks of culture the relaxin 2 transfectants created significantly more colonies when compared to EGFP control in all three cell confluences (A). Pictures of soft agar test (B).

4.3.8 Relaxin 2 in nude mice

To investigate whether relaxin 2 can alter tumour growth of human thyroid carcinoma cells, the nude mice were used as *in vivo* models for the solid tumour xenotransplantation.

First tumours with relaxin 2 transfected cells had already developed after one week in both relaxin 2 (FTC-133-RLN2 cl.4 and FTC-133-RLN2 cl.10), but not in FTC-133-EGFP injected animals. The mean volume of the visible part of tumours at the first week reached 4,4 mm^3 for the FTC-133-RLN2 cl.4 and 0,4 mm^3 for the FTC-133-RLN2 cl.10 in the second week. Three weeks after injection, the mean volume of the visible part of FTC-133-RLN2 cl.4 attained 1258,5 mm^3, and after four weeks clone 10 attained 1023 mm^3. The FTC-133-RLN2 cl.4 animals were sacrificed

three weeks after injection due to the rapid growth of tumours. The animals injected with FTC-133-RLN2 cl.10 were sacrificed five weeks after injection. In all cases, pathological investigation and X-ray failed to detect metastases.

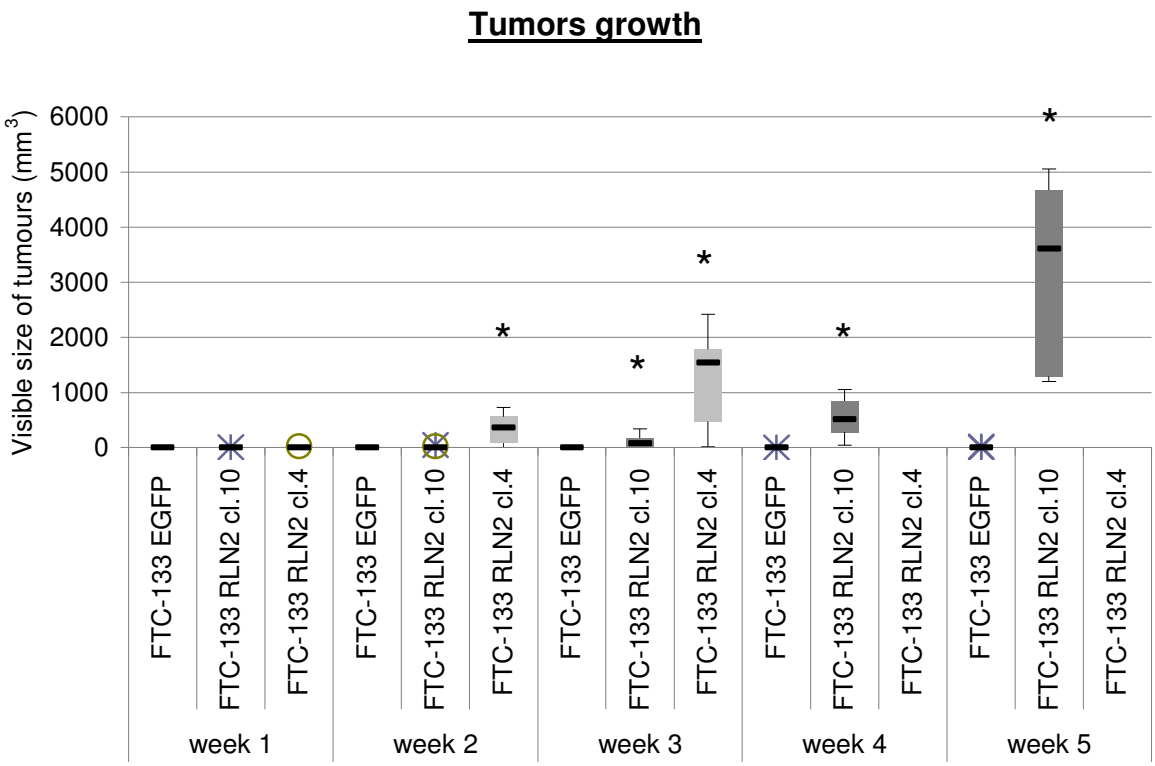


Figure 25: Tumor growth *in vivo*. Relaxin 2 clones developed significantly bigger tumours after injection in NMRI nude mice when compared with EGFP control.

Histological analysis revealed relaxin-2-induced formation of encapsuled xenograft tumours. Microscopic analysis displayed a high number of mitotic cells in relaxin 2 induced tumours, and hematoxylin-eosin staining showed high vascularisation of those tumours.

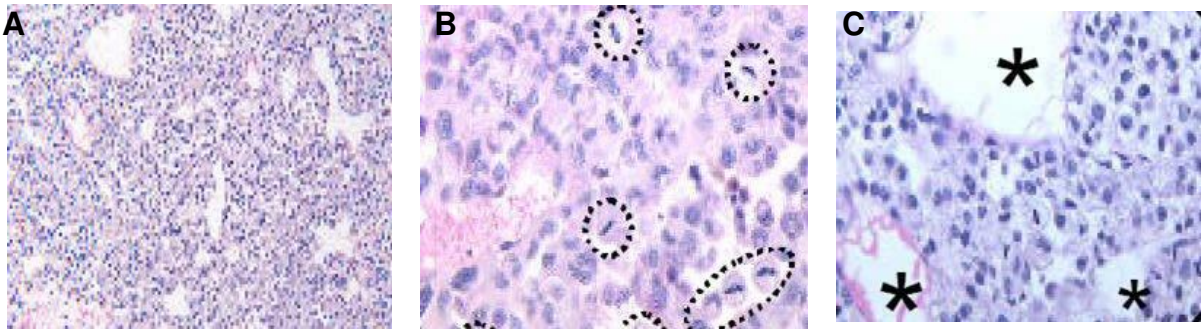


Figure 26: Microscopical pictures of FTC-133-RLN2 tumours. Hematoxylin/Eosin staining (A) revealed high number of mitotic cells (B) and blood-vessels (C).

5 Discussion

In this study we identified human thyroid carcinoma tissues, but not normal or hyperplastic, as a potential source of relaxin 2. Presence of relaxin 2 receptor RXFP1 in all tested patient samples (neoplastic and non-neoplastic) makes the thyroid as a potential target for autocrine and paracrine activity of relaxin 2. Previous investigations of our group revealed expression of relaxin 2 in neoplastic interstitial C-cells suggesting its role in medullary thyroid carcinogenesis (Klonisch et al., 2005). Apart of the RXFP1 we have also found the expression of the second relaxin 2 receptor RXFP2 and its main ligand INSL3 in human thyroid tissues and thyroid carcinoma cell lines (Hombach-Klonisch et al., 2003).

As a model to analyse the function of relaxin 2 in neoplastic thyrocytes we established stable transfectants using human follicular thyroid carcinoma cell lines FTC-133 and FTC-238. We could detect only the proform of relaxin 2 in both transfected cell lines, so we deduce that the capacity of transfectants to process the relaxin 2 to matured form is limited. In our other studies human thyrocytes produced only the proform of relaxin-like protein INSL3 (Bialek et al., 2009), what can suggest that generally the capability of proceeding relaxin-like proteins in thyroid is limited. The bioactivity of proform of relaxin 2 was reported also in other systems (Silvertown et al., 2003b, Vu et al., 1993, Zarreh-Hoshari-Khah et al., 2001). Pro-relaxin 2 produced by transfectants induced cAMP accumulation, which confirmed the potential of the hormone to activate the receptor. The specifity of ligand-receptor interactions in transfected cells was proved using siRNA construct. Down-regulation of the receptor expression reduced response to relaxin 2 treated cells as demonstrated by lower cAMP accumulation and indicating RXFP1 as a mediator of relaxin 2 actions in these thyroid carcinoma cells.

In our investigations we demonstrate the role of relaxin 2 in regulation of cell metabolismus, displayed by increased mitochondrial activity and in intracellular production of ATP in RLN2 clones. Masini et al. previously demonstrated the role of relaxin 2 in mitochondria of cardiomyocytes in hypoxic conditions showing the ability of relaxin 2 to prevent swelling (Masini et al., 1997). Like in endometrial cancer (Kamat et al., 2006), in thyroid carcinoma cells relaxin's 2 mitotic activity was not

increased, but the high number of colonies during anchorage-independent growth suggests the role of the hormone in cellular viability and tumour cell survival.

In RXFP1-dependent manner relaxin 2 modulates migration of the thyroid carcinoma cells. Silvertown et al. have previously described induction of cell motility in canine CF33.MT (Silvertown et al. 2003b) and Wyatt et al., and Unemori et al., in non-carcinoma bronchial epithelial cells and inflammatory cells (Unemori et al., 2000, Wyatt et al., 2002). We introduced relaxin 2 as an inductor of motility of follicular thyroid carcinoma cells FTC-133 and FTC-238. Our data demonstrated that both recombinant and transfectant-secreted relaxin 2 increase motility of the FTC-133 and FTC-238 cells in paracrine and autocrine manner. Also other members of relaxin family demonstrated the ability to influence cell motility, for example INSL3 enhances the motility of human thyroid carcinoma cells FTC-133 (Bialek et al., 2009) and prostate carcinoma cell line PC-3 (Klonisch et al., 2005).

Motility of cells is complicated and depends on many factors. We demonstrated that relaxin 2 changed the morphology of FTC-133 cells towards a fibroblast-like phenotype in both, transfectants and FTC-133 wild type cells incubated with recombinant relaxin 2. It is worth nothing that such morphological changes are typical for cells with increased metastatic potential (Cai et al., 2009).

Morphological changes of the cells are induced by the changes in cytoskeletal architecture. The cytoskeleton is a dynamic structure that maintains cell shape, enables cellular motility and plays important roles in both intracellular transport and cellular division. Actin is directly regulated by cofilin. Cofilin is an actin-binding protein and the main player in actin turnover, however, it also may affect actin filaments (Ghosh, et al., 2002). The consequence of actin and cofilin actions is cytoskeleton reorganisation (Carlier et al., 1999). We have identified cofilin as one of the potential actin modulators in thyroid carcinoma cells. We found that changes in cofilin activity and actin organisation lead to the increased motility of the cells. Studies employing insulin-like growth factor I (I-IGF I) showed that cofilin induced the motility of neuroblastoma cells through PI-3K, Rac and LIMK pathways upon IGF stimulation (Meyer et al., 2005). Other reports demonstrated cofilin as a modulator of B16F1 melanoma cells morphology. Silencing of cofilin in those cells resulted in larger and flattened non-polarised cells, creating lamelipodia in different directions and shapes, which slowed the cells' locomotion (Hotulainen et al., 2005). In our studies we observed the alterations in cofilin production and its phosphorylation. We found that

transfectants stably expressing increased levels of relaxin 2 revealed higher production of total and phosphorylated cofilin.

Many studies indicated that phosphorylation inactivates cofilin (Carlier et al., 1999, Yamaguchi et al., 2007). Yamaguchi et al, however, observed phosphorylation but not inactivation of total cofilin within the cells. It is also postulated that during stimulation of cell migration cofilin coexists as two populations – one locally activated initiating localised protrusions and the second phosphorylated to recycle cofilin or to confine its activity (Yamaguchi et al., 2007). Stimulation of tumour cells with EGF increases the migration rate of the cells as a consequence of cofilin activation (Yamaguchi et al., 2007). It is known that stimulation of EGFR activates PLC, which releases cofilin from the inactive complex with PIP2, but on the other hand induces its phosphorylation by LIMK. The balance between both processes is crucial for the motility of the cells (Song et al., 2006, Yamaguchi et al., 2007). Moreover, the localisation of the protein is important. It was shown that although after EGF stimulation the total amount of phosphorylated cofilin is increased, most of it was localised in the centre and only small amounts were accumulated on the leading edges of the cells (Song et al., 2006, Yamaguchi et al., 2007).

In the thyroid both form of cofilin are present. Thyrotropin (TSH) is one of the factors mediating dephosphorylation of cofilin in these cells (Saito et al., 1994). This process can be implicated in disruption of actin containing stress-fibers and reorganisation of actin filaments (Saito et al., 1994). Clinical and pathological evidences revealed increased levels of cofilin in more aggressive variant of papillary thyroid carcinoma tissues (Giusti et al., 2008), characterised by elongated shape of the cells (Ghossein et al., 2008). We found that relaxin 2 induced elevated production and phosphorylation of cofilin what coincided with morphological alternations of thyroid carcinoma cells and increased motility. Although we can not exclude the participation of other factors, relaxin 2-induced changes in cytoskeleton organisation may be partially mediated by cofilin.

Migration of the cells and especially degradation of extracellular matrix are some of the crucial processes involved in cancer progression and invasiveness. Several reports demonstrated that relaxin 2 regulates the expression and activity of MMPs and their physiological inhibitors – TIMPs (Kamat et al., 2006, Khasigov et al., 2003, Kraiem et al., 2000). In human MCF-7 and SK-BR3 cells, Binder et al. demonstrated that relaxin 2-induced migration of the cells coincided with relaxin 2 stimulated

MMP-activity (Binder et al., 2002). Other reports illustrated the correlations between increased levels of relaxin 2 in serum and metastases of patients with breast cancer (Binder et al., 2004). In canine mammary carcinoma cells, relaxin 2 mediated induction of MMPs and increased migration of the cells which correlated with ECM degradation (Silvertown et al., 2003b). We demonstrate in our studies that relaxin 2-mediated degradation of ECM affected the production of two ECM components, collagen and elastin. Furthermore, we found that relaxin 2 actions coincided with elevated levels of several proteases. We found that Cathepsin L and Cathepsin D are novel targets of relaxin 2 in thyroid carcinoma cells. Previous reports demonstrated the involvement of Cathepsin D in thyroid tumour growth and metastasis (Métayé et al., 1997), indicating that concentrations of Cathepsin D correlate with tumour size (Kraimps et al., 1995), and described this protein as a marker of proteolytic activity during invasion of thyroid carcinoma (Métayé et al., 1993). Métayé et al. detected increased levels of Cathepsin D in carcinomas and toxic adenomas when comparing with normal tissues, cold benign nodules and Graves' disease tissues (Métayé et al., 1997). Results obtained on thyroid cell lines FTC-133 and 8505C suggested participation of Cathepsin L in aggressive behaviour of the cells (Plehn et al., 2000). We demonstrated that relaxin 2 induced elevated production and secretion of Cathepsin L which correlated with increased ability of thyroid carcinoma cells to penetrate elastin matrix. Previous investigations performed on tumour cells displayed Cathepsin L as a promoter of migration and basement membrane degradation *in vitro* (Jedeszko et al., 2004, Krueger et al., 2001, Novinec et al., 2007) and *in vivo*. Kirschke et al. noticed that after silencing of Cathepsin L, malignant cells developed no or only small tumours (Kirschke et al., 2000). Similar results were obtained by Dunn et al. in experiments *in vivo*, where Cathepsin L was associated with tumour growth and invasion (Dunn et al., 1991). Clinical investigations of gastric carcinomas revealed correlation between expression of Cathepsin L and venous invasion (Dohchin et al., 2000). In breast cancer Cathepsin L is proposed as a strong independent prognostic factor and was associated with lymph node status and tumour grading and staging (Thomssen et al., 1995). Accompanied by Cathepsin B, Cathepsin L is associated with shorter disease-free survival rates for breast cancer patients (Jedeszko et al., 2004). We found that in thyroid carcinoma cells relaxin 2 additionally promoted changes in cytosolic distribution of Cathepsin L. Polar localisation of

Cathepsin L induced by relaxin 2, detected only in relaxin 2 but not mock control transfectants coincided with similar changes in distribution of mannose-6-phosphate receptor. Interestingly, no alterations in localisation of Cathepsin D were detected. This finding suggests specific, relaxin 2-dependent trafficking of Cathepsin L. Additionally, we found that relaxin 2 induced increased production of Cathepsin D what is in agreement with previous studies demonstrating that concentrations of Cathepsin D are higher in thyroid carcinoma tissues and correlate with tumour size and stage (Kraimps et al., 1995).

For the first time we showed the correlation between relaxin 2 and expression of metalloproteinases in thyroid carcinoma cells. Previously mentioned cathepsins are demonstrated to cooperate with other peptidases, like MMPs, during tumour progression or invasion (Jedeszko et al., 2004). Our data demonstrate relaxin 2 as a modulator of metalloproteinases in follicular thyroid carcinoma cell line FTC-133. We detected increased expression of MMP-2 and MT1-MMP in FTC-133 clones stably producing relaxin 2. Previous reports demonstrated the influence of relaxin 2 on the metalloproteinases activity not only in thyroid tissues but also in breast cancer (Binder et al., 2002) or endometrial cancer (Kamat et al., 2006). The increased expression of MMP-2 or MT1-MMP in thyroid carcinomas was already reported (Cavalheiro et al., 2008, Nakamura, et al., 1999; Yeh et al., 2006), however, the mechanism of relaxin 2-mediated regulation of MMPs remains to be clarified.

Relaxin 2-induced collagenolytic activity of transfected FTC-133 cells underlines the role of the hormone in increased ability of transfectants to degrade ECM. Collagens are important components of basement membrane (Monaco et al., 2006) and stroma (Ohuchi et al., 1997) and their degradation facilitate the cells to overcome the invasion barrier.

Previously relaxin 2 was shown to induce migration and invasion of endometrial cancer cells HEC-1B and KLE by enhancing the activity of MMP-9 and MMP-2, respectively (Unemori et al., 1996). The metalloproteinases play an important role in invasion process of breast carcinoma (Binder et al., 2002), and gastric cancers (Nomura et al., 1995). Expression and activation of MT1-MMP and MMP-2 was demonstrated also in thyroid cancer where carcinoma tissues showed increased production of proMMP-2 when compared to the follicular adenoma and normal sites obtained from carcinoma-affected gland. Additionally, all carcinomas with lymph node metastasis displayed increased MMP-2 activity, which correlated with expression of

MT1-MMP (Nakamura et al., 1999). In other clinical studies MMP-2 immunostaining associated significantly with extra-thyroidal invasion, lymph node metastasis and depth of tumor invasion (Tian et al., 2008). High expression of MMP-2 in follicular carcinomas revealed useful predictive potential to discriminate between follicular carcinoma and adenoma (Cho Mar et al., 2006).

Previously, a direct correlation between MT1-MMP expression and lymph node metastasis of PTC was shown (Nakamura et al., 1999). In other studies, performed on PTC tissues increased MMP-2 expression was accompanied by elevated levels of VEGF-C and they were significantly more frequently observed in PTC with lymph node metastases than without. This finding suggests both MMP-2 and VEGF-C as possible tumour markers for metastatic PTC (Tian et al., 2008).

Increased expression of MT1-MMP was also associated with invasion of breast carcinoma cells *in vitro* (Jiang et al., 2006, Koshikawa et al., 2000) and *in vivo* (Jiang et al., 2006, Mimori et al., 2001). Prognostic value of MT1-MMP was also established in gastric cancer patients, where its increased expression served as an indicator of distant metastasis (Mimori et al., 2008).

We could demonstrate that follicular thyroid carcinoma cells with increased expression of relaxin 2 showed an anchorage-independent growth and in xenograft models relaxin 2 induced rapid growth of highly vascularised tumours. However, no local or distant metastases were found. This could be the effect of subcutaneous injection. As well as in tumour development, the surrounding environment plays a crucial role in metastasis. In natural conditions, tumour development in the thyroid takes place in gland-specific ECM and consists of neoplastic and host cells such as fibroblasts, lymphocytes, macrophages and dendritic cells as the immune response, which are crucial for invasion and metastasis (Kawashiri et al., 1995, Liotta et al., 2001, Pocard et al., 2001).

6 Conclusion

In this work, we demonstrated the role of relaxin 2 in promoting the aggressive character of follicular thyroid carcinoma cells. Increased expression of relaxin 2 in thyroid carcinomas indicates the hormone as important molecule in development of tumours. Our data obtained from human thyroid carcinoma cell lines FTC-133 and FTC-238 demonstrate the ability of relaxin 2 to induce aggressive behaviour of thyroid epithelial cells. We identified proteolytic enzymes Cathepsins L and D, and MMP-2 and MT1-MMP as novel executors of relaxin 2-mediated actions. Although we can not exclude an involvement of other factors, relaxin 2 by active participating in morphological changes of the cells and elevation of proteolytic activity may promote thyroid carcinoma progression. Relaxin 2 may be considered as a new, additional diagnostic marker for human thyroid carcinoma.

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Selbständigkeitserklärung

Gemäß §6 der Promotionsordnung der Universität Halle erklärte ich hiermit an Eides statt, dass ich vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Frühere Promotionsversuche bestehen nicht. Die vorliegende Arbeit wurde weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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Publications

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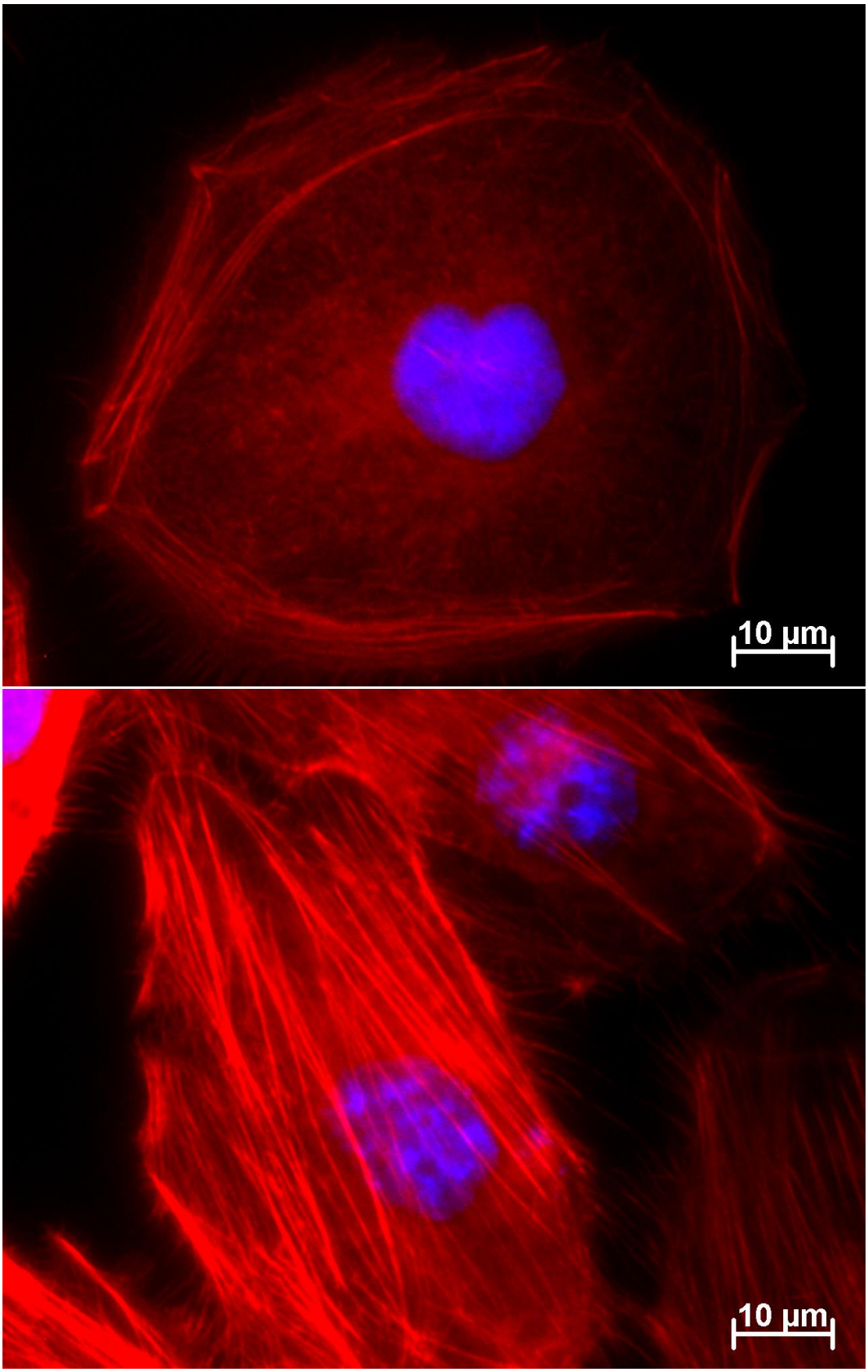
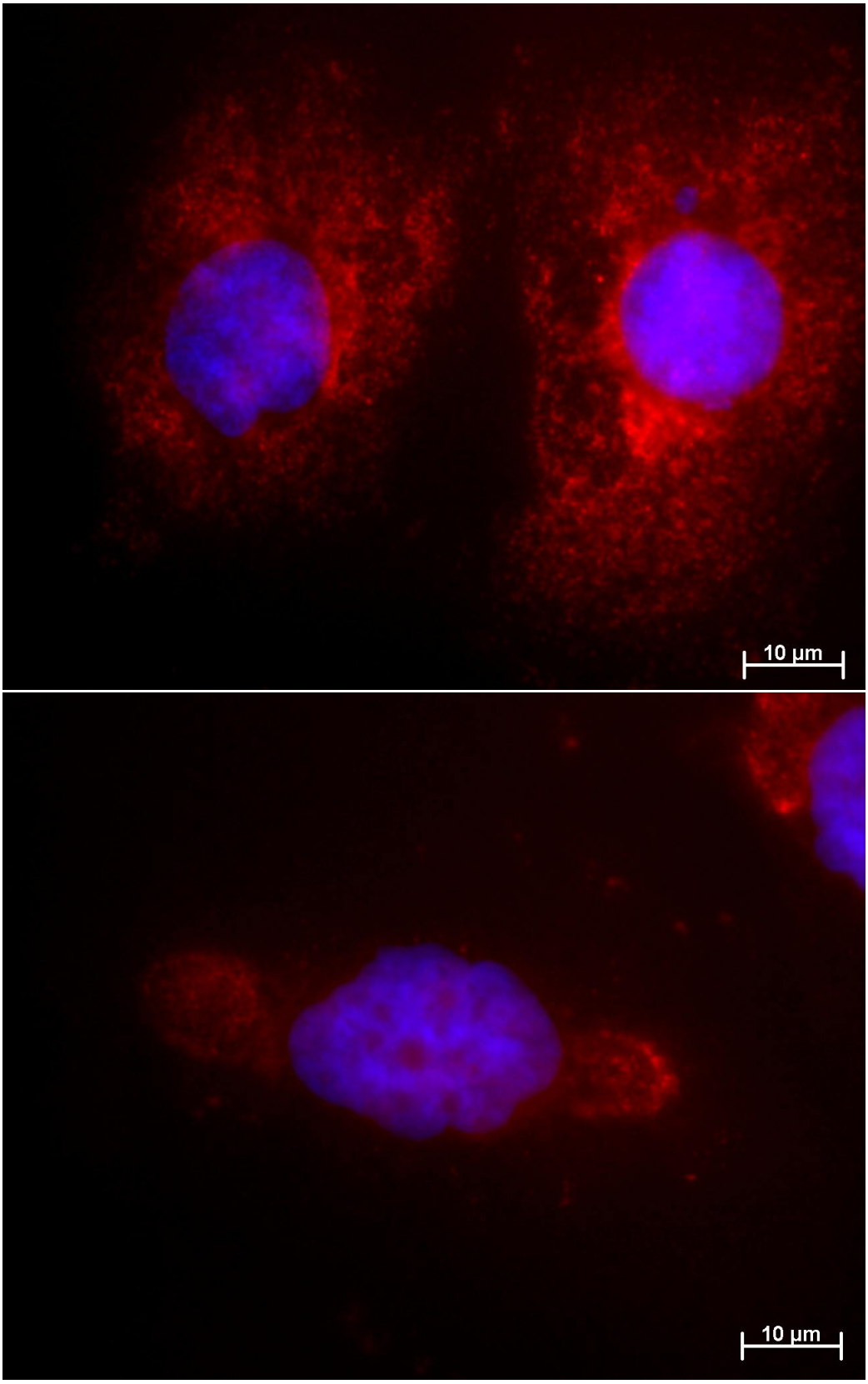
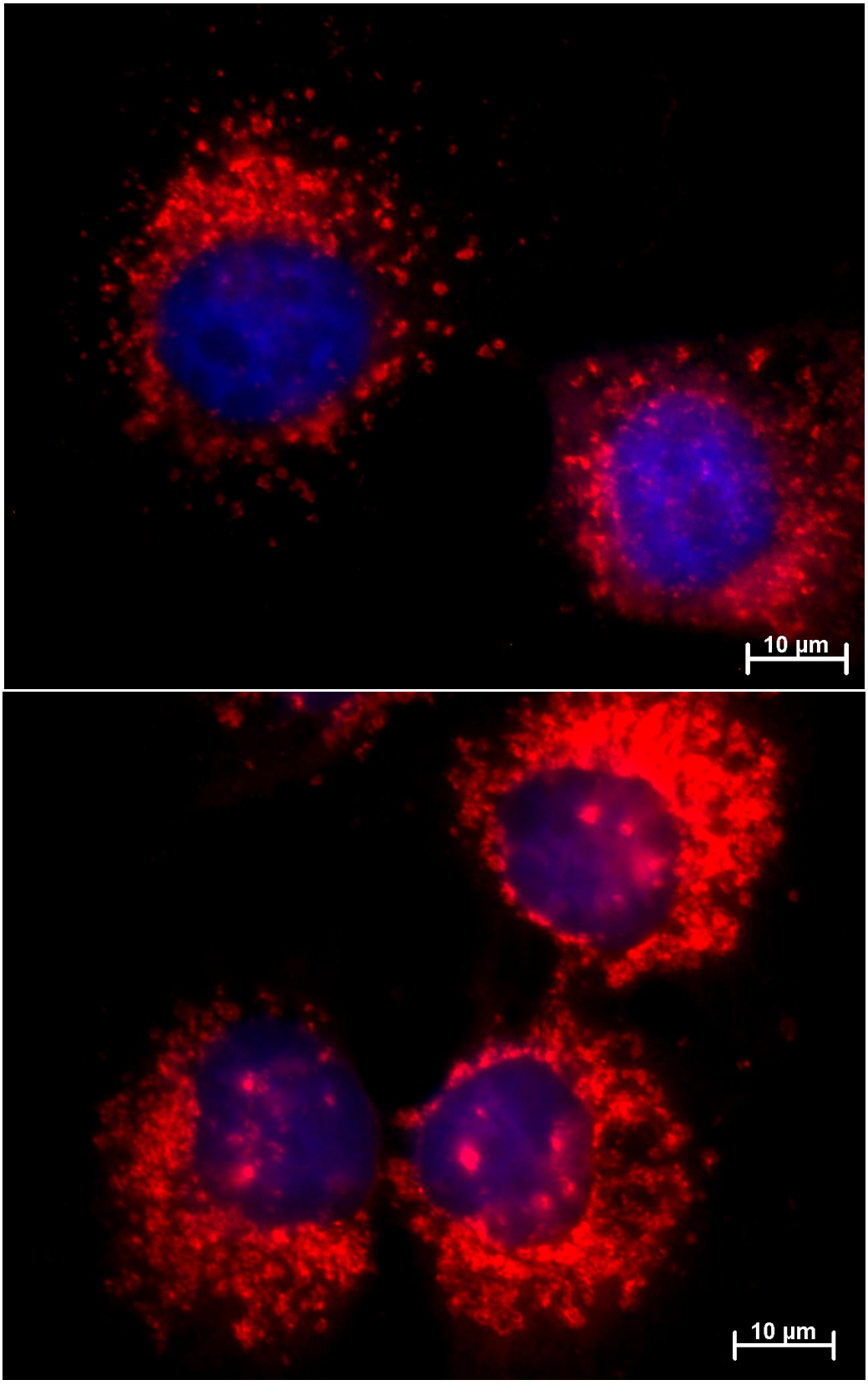
Attachment



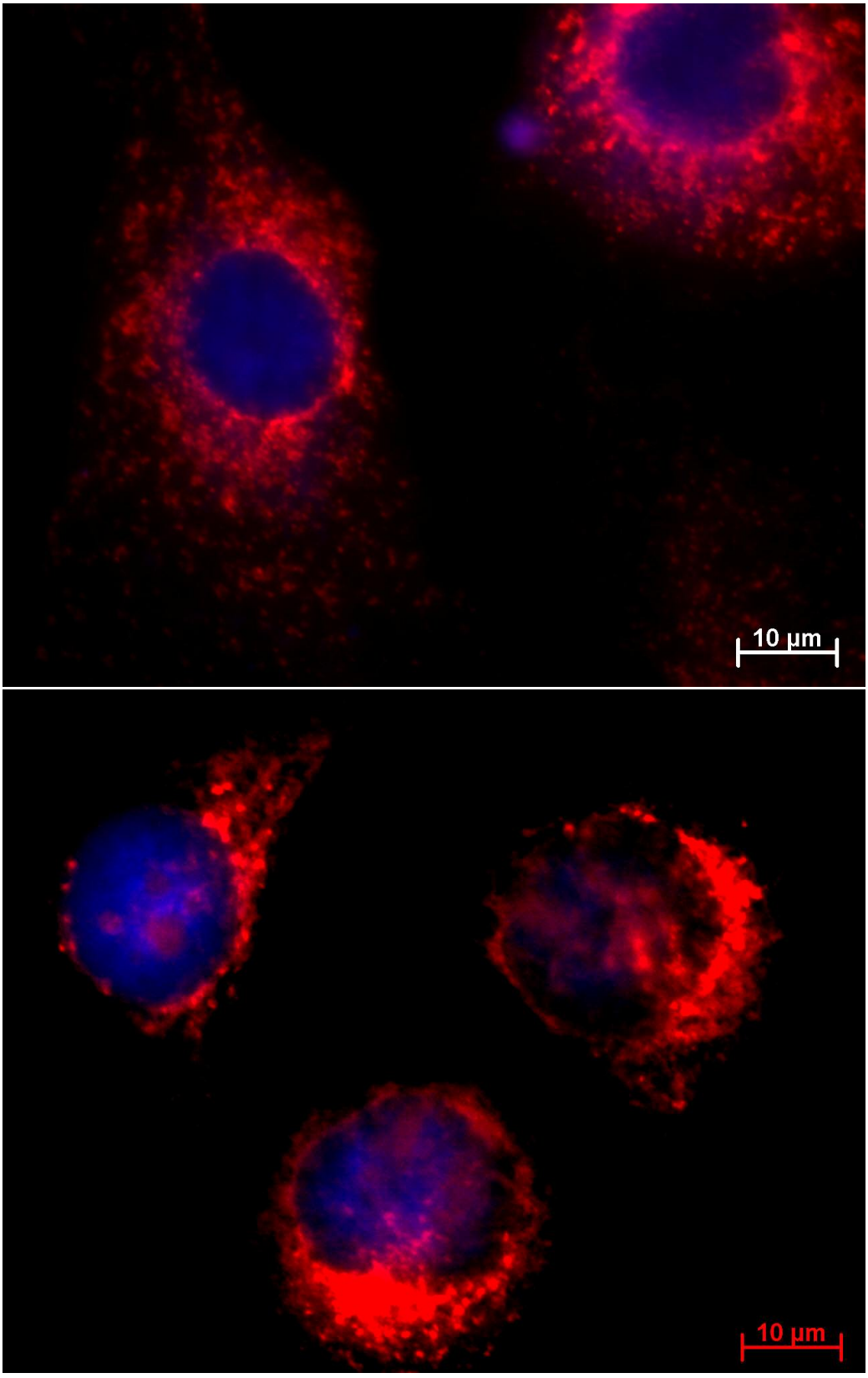
Magniffication of figure 12 (page 59), displaying F-actin localization in FTC-133 mock (upper) and relaxin (lower) transfectants



Magniffication of figure 16 (page 63), displaying Cathepsin L localization in FTC-133 mock (upper) and relaxin (lower) transfectants



Magniffication of figure 16 (page 63), displaying CD63 localization in FTC-133 mock (upper) and relaxin (lower) transfectants



Magniffication of figure 16 (page 64), displaying M6PR localization in FTC-133 mock (upper) and relaxin (lower) transfectants