NOD proteins and their functional relevance to normal and adenomatous pituitary

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Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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Abstract

During infection or inflammation pituitary hormone production is altered in a specific manner to adapt the endocrine system to the activated immune system. One major class of innate receptors, the NOD-like receptors, are known as PRRs (pattern recognition receptors) and involved in the early immune response upon bacterial infection through the activation of downstream signaling pathways, including NF-κB (nuclear factor kappa B) and other important signaling pathways. Here, we investigated the expression and activation of NOD (nucleotide oligomerization domain) immune receptors in AtT20 pituitary tumor cells and in pituitary adenomas. Moreover, it could be hypothesized that the PI3K/AKT pathway plays a role in NOD signaling-induced pituitary tumor survival. As shown, NOD-like receptors 1 and 2 (NOD1, NOD2) are expressed in the pituitary gland and in AtT20 cells. Increased mRNA levels of NOD1 and NOD2 could be observed in a subset of human pituitary adenomas in comparison with normal pituitaries. In AtT20 cells and human pituitary adenomas, NOD1 ligand, diaminopimelic acid and NOD2 ligand, muramyl dipeptide, induced proliferation. In contrast, the proliferative effect of NOD ligands was abolished in AtT20 cells treated with PI3K/AKT inhibitors, LY294002 and Wortmannin, and NF-κB inhibitor CAPE (Caffeic acid phenethyl ester). Further, NOD ligands activated several components of the PI3K/AKT pathway in AtT20 cells such as AKT, mTOR and p70S6K1. In contrast, they downregulated tumor suppressors such as PTEN, p27, p53 and BAX pro-apoptotic gene expression. Moreover, NOD ligands increased Cyclin D1, D3 and E protein expression. In the present study it is demonstrated that NOD activation promotes the survival of AtT20 cells through several signaling pathways. These findings provide a new insight into infection-associated tumorigenesis and illustrate the importance of immune receptors in the pathophysiology of pituitary tumoral cells. NOD receptors might be a new therapeutic target in the prevention of tumor progression.

List of abbreviations

A

Ab
Antibody ·
ABC
Avidin-Biotin-Complex ·
ACRO
Acromegaly ·
ACTH
Adrenocorticotropin hormone ·
Ag
Antigen ·
AP-1
Activator protein-1 ·
APCs
Antigen-presenting cells ·

B

BCL-2
B cell lymphoma 2 ·
BS
Blau Syndrom ·
BSA
Bovine serum albumin ·

C

CAPE
Caffeic acid phenethyl ester ·
CARD
Caspase recruitment domain ·
CD
Crohn`s Disease ·
CDK
Cyclin-dependent kinase ·
CKIs
Cyclin-dependent kinase inhibitors ·
cpm
counts per minute ·
CRH
Corticotropin-releasing hormone ·
CUSH
Cushing ·

D

DAB
Diaminobenzidine ·
DEPC
Diethyl-pyrocarbonate ·
DMEM
Dulbecco`s Modified Eagle Medium ·
DMSO
Dimethylsulfoxid ·
dsRNA
double-stranded Ribonucleic Acid ·
DTT
Dithiothreitol ·

E

EDTA
Ethylenediaminotetracetic acid ·
ERK
Extracellular-signal-regulated kinase ·

F

FCS
Fetal Calf Serum ·
Fig
Figure ·
FOXO
Forkhead Box O ·
FSH
Follicle-stimulating hormone ·

G

GAPDH
Glycerinaldehyd-3-phosphat-Dehydrogenase ·
GH
Growth hormone ·
GPCRs
G-protein-coupled receptors ·
GSK-3β
Glycogen synthase kinase-3β ·

H

HIF-1α
Hypoxia-inducible factor-1α ·
HPA
Hypothalamic-Pituitary-Adrenal ·

HSPs
Heat Shock Proteins ·

I

IAP
Inhibitor of Apoptosis Protein ·
iE-DAP
γ-D-glutamyl-meso diaminopimelic ·
IGF-I
Insulin-like growth factor I ·
IHC
Immunohistochemistry ·
IKK
IκB kinase ·
IL
Interleukin ·

J

JNK
c-Jun NH2-terminal kinase ·

L

LH
Luteinizing hormone ·
LPS
Lipopolysaccharide ·
LRRs
Leucine-rich repeats ·
Luc
Luciferase ·

M

MAPK
Mitogen-activated protein kinase ·
MDM2
Murine double minute 2 ·
MDP
Muramyldipeptide ·
mRNA
messenger Ribonucleic Acid ·
mTOR
mammalian Target of Rapamycin ·
mTORC1
mTOR complex 1 ·

N

NEMO
: NF-κB essential modulator ·

NFPA
: Non functioning pituitary adenoma ·

NF-κB
: Nuclear factor kappa B ·

NLR
: Nod-like receptor ·

NOD
: Nucleotide oligomerization domain ·

NP
: Normal pituitary ·

O

ONPG
: O-nitrophenyl-b-D-galactoside ·

P

PAMPs
: Pathogen-associated molecular patterns ·

PBS
: Phosphate buffered saline ·

PCR
: Polymerase Chain Reaction ·

PDK-1
: Phosphoinositide-dependent kinase-1 ·

PFA
: Paraformaldehyde ·

PGN
: Peptidoglycan ·

PH
: Pleckstin homology ·

PI3K
: Phosphoinositide 3-kinase ·

POMC
: Proopiomelanocortin ·

PRL
: Prolactin ·

PRRs
: Pattern recognition receptors ·

PTEN
: Phosphatase and tensin homolog deleted at chromosome ten ·

PVDF
: Polyvinylidene fluoride ·

R

Rb
Retinoblastoma ·
RHD
Rel homology domain ·
RIA
Radioimmunoassay ·
RICK
Rip-like interactive clarp kinase ·
RIP2
Receptor interacting protein-2 ·
RISC
RNA induced-silencing complex ·
RNA
Ribonucleic Acid ·
RNAi
Ribonucleic Acid interference ·
rpm
revolutions per minute ·
RSV-β-gal
Rous Sarcoma Virus-β -galactosidase ·
RTKs
Receptor tyrosine kinases ·
RT-PCR
Reverse Transcriptase-Polymerase Chain Reaction ·

S

SDS-PAGE
Sodium dodecyl sulfate polyacrylamide gel electrophoresis ·
siRNAs
small interfering Ribonucleic Acids ·

T

TAK1
TGFβ-activated protein kinase 1 ·
TB
Tris buffer ·
TBE
Tris borate EDTA ·
TBS
Tris-buffered saline ·
TBST
Tris-buffered saline tween-20 ·
TLR
Toll like receptor ·
TNF-α
Tumor necrosis factor alpha ·
TSH
Thyroid-stimulating hormone ·

V

VEGF
: Vascular endothelial growth factor ·

VIP
: Vasoactive intestinal peptide ·

W

WST
: Water soluble tetrazolium ·

X

XIAP
: X-linked Inhibitor of Apoptosis Protein ·

1. Introduction

1.1 The pituitary gland

The pituitary gland is located at the base of the brain beneath the hypothalamus, and is no larger than a pea. It is an important component of the endocrine system, and together with the hypothalamus, exerts considerable influence over the functions of other endocrine glands. The hypothalamus either positively or negatively regulates hormonal production in the pituitary through its release of various hormones, which act on specific cell types in the pituitary to secrete a variety of pituitary hormones, which are important for growth and development, metabolism, reproductive and nervous system functions. The pituitary (Fig. 1) is composed of two different lobes: the anterior lobe or adenohypophysis, that derives embryologically from an evagination of the oral cavity (Rathke´s pouch), and the neuronal part also known as posterior lobe or neurohypophysis, derived by a downward extension of the hypothalamic area, which forms the hypophyseal stalk. Between the anterior and posterior lobes there is a third part, the intermediate lobe, which in rodents plays a significant role in the regulation of pigmentation, but in humans is reduced to a few cells without any known function.

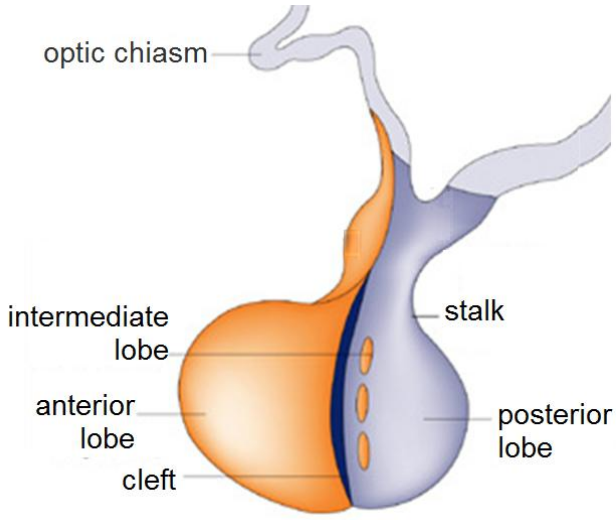


Fig. 1: The pituitary gland. The normal pituitary is a bean-shaped gland that has two components. The neurohypophysis, also known as the posterior lobe, is an extension of the hypothalamus. The adenohypophysis is composed of hormone-secreting epithelial cells, known as adenohypophysial cells, which are derived from the oral ectoderm and ascend as Rathke's pouch during development. The intermediate lobe, or pars intermedia, is composed of epithelial cells from the posterior limb of Rathke's pouch and is only rudimentary in humans.

The anterior lobe produces the following hormones, which are regulated by the hypothalamus:

- Growth hormone - GH promotes growth of the skeleton and soft tissues and has important metabolic effects. It acts directly on peripheral GH receptors or indirectly by inducing insulin-like growth factor I (IGF-I) synthesis in the liver.
- Thyroid-stimulating hormone (TSH) - stimulates the thyroid gland to produce thyroid hormones.
- Adrenocorticotropin hormone (ACTH) - ACTH induces glucocorticoid secretion from adrenal cortex and is a split product of proopiomelanocortin (POMC), a precursor protein from which also endorphins, encephalins, corticotropin-like immunoreactive peptides and melanocyte stimulating hormones are produced.
- Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) - hormones that control sexual function and production of the sex steroids estrogen and progesterone in females or testosterone in males.
- Prolactin - hormone that stimulates milk production in females.

Generally, 50% of the cells are somatotropes, 10%–20% are lactotropes, 10%–20% are thyrotropes, 10%–20% are corticotropes and 10%–20% are gonadotropes [1]. The posterior lobe produces the following hormones, which are not regulated by the hypothalamus:

- Antidiuretic hormone (vasopressin) - controls water loss by the kidneys.
- Oxytocin - contracts the uterus during childbirth and stimulates milk production.

The hormones secreted by the posterior pituitary are actually produced in the brain and carried to the pituitary gland through nerves where they are stored.

1.2 Pituitary tumor cells AtT20 and their endocrine applications

This cell line was established by alternatively passaging ACTH-producing mouse pituitary tumors in culture and in animals [2]. ACTH-producing tumor cells were first dispersed and cultured, and after various periods in culture, were injected into mice to obtain new tumors. New tumors which still produced ACTH were then put back into culture, and the process repeated to select cell lines that are able to produce ACTH and grow in culture permanently [3]. Like the corticotropes in the anterior pituitary, AtT20

cells synthesize POMC, the precursor to ACTH and endorphins, and correctly process it to the mature forms of the hormones before they are secreted. Consequently, this cell line was used extensively to study the processing of POMC and how this process is regulated by glucocorticoids and cytokines [4-11]. Corticotropin releasing hormone (CRH), vasoactive intestinal polypeptide (VIP), phorbol ester, forskolin, IL-1 and IL-6 regulate the secretion of ACTH and β-endorphin from the AtT20 mouse pituitary cell line [12-13]. AtT20 cells express glucocorticoid receptors [14-18], somatostatin receptors [19], interleukin-1 receptors [20-22], high affinity histamine H3 receptors [23-24], dopamine receptors [25-27], as well as muscarinic cholinergic receptors [28-30], all of which can regulate ACTH release.

1.3 Tumor development and cell cycle – a general overview

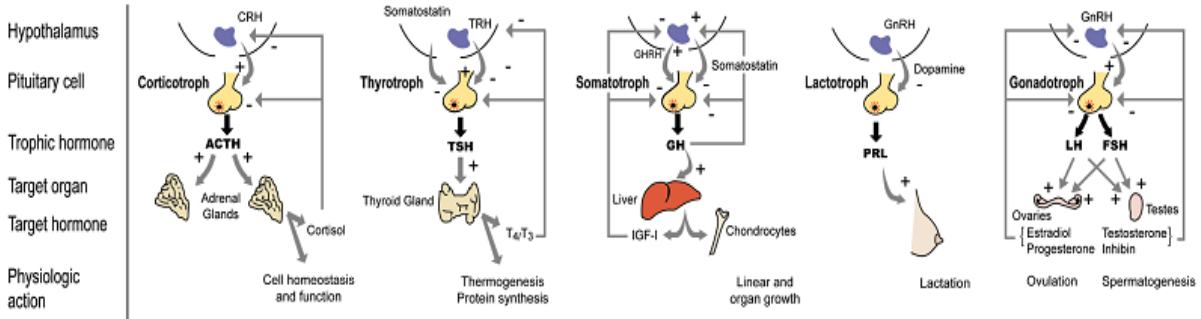
1.3.1 Tumor development

Cancer is a hyperproliferative disorder that involves morphological cellular transformation, dysregulation of apoptosis, uncontrolled cellular proliferation, invasion, angiogenesis and metastasis. Clinical and epidemiologic studies have suggested a strong association between chronic infection, inflammation and cancer. The vast majority of cancer cells harbor six common properties that are essential for the transformation of a normal cell into a malignant phenotype [31]. The acquired capabilities, such as self-sufficiency in growth-signals, insensitivity to anti-growth signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, mediate the successful breakage of the pre-existing anticancer defense mechanism of the organism. These changes involve activation of oncogenes and inactivation of tumor suppressors, leading to an imbalance between pro- and anti-growth signaling [32]. Tumor development and growth are thought to be the outcome of a series of different mutations occurring in a somatic cell, leading to progressive acquisition of proliferative advantage compared to non-mutated cells. These mutations can affect different genes essential for cell survival, belonging to two major categories: proto-oncogenes and tumor suppressor genes. Proto-oncogenes have an important role in the regulation of cell proliferation, differentiation and apoptosis. They usually act as positive regulators of cell growth and in cancer they can be activated by gain of function point mutations, overexpression because of gene amplification, translocation of the

proto-oncogene in an area of actively transcribed chromatin, increase in promoter activity or protein stability. On the other hand, tumor suppressor genes inhibit cell proliferation and are mostly involved in the inhibition of cell cycle progression, induction of cell differentiation and programmed cell death and in the assembly of the mitotic machinery. They acquire transforming potential by losing their anti-proliferative properties. This can be due to loss of function mutations, loss of a big chromosomic portion or to epigenetic alterations like hypermethylation of cytosine-guanine dinucleotides in promoters with consequent gene silencing [33].

Pituitary adenomas

Pituitary adenomas are benign neoplasms, accounting for approximately 15% of intracranial tumors. An occult adenoma is discovered in about 25% of unselected autopsies. Pituitary tumors are usually benign and do not metastasize, although some of them can become invasive, leading to bone destruction and infiltration within the cavernous sinus. They can cause severe clinical symptoms due to their critical location and expanding size, like headache and visual disorders, and to inappropriate pituitary hormone production. The clinical features associated with hormonal hypersecretion reflect specifically the type of endocrine cell from which the adenoma arises. Therefore, ACTH oversecretion results in Cushing disease with features of hypercortisolism, GH hypersecretion leads to acral overgrowth and metabolic dysfunction associated with acromegaly and PRL hypersecretion leads to gonadal failure, secondary infertility and galactorrhea. More rarely, TSH hypersecretion leads to hyperthyroxinemia and goiter, and hypersecreted gonadotropins lead to gonadal dysfunction. Non functioning pituitary adenomas (NFPA), also known as hormone inactive, do not secrete hormones and therefore have no typical hormone excess-related presentation. An overview of the secreted hormones of the pituitary gland and the pituitary adenomas is given in figure 2.



	Cushing's Disease	TSH-cell Adenoma	Acromegaly	Prolactinoma	Non-functioning Adenoma
Tumor phenotype	Hypercortisolism	Thyroid goiter	Acral enlargement	Galactorrhea	Central effects
	Central obesity	Hyperthyroxinemia	Soft tissue swelling	Amenorrhea	Hypogonadism
	Striae		Cardiac hypertrophy	Infertility	Hypergonadism (rare)
	Hyperglycemia		Hypertension	Hypogonadism	Clinically silent
	Osteoporosis		Hyperglycemia		
	Hirsutism		Sleep apnea		

Fig. 2: Hypothalamic-pituitary regulation and pituitary tumor pathogenesis

Pituitary tumor development results from both intrinsic alterations of the pituitary gland cells and from dysregulation of regulatory factors like hypothalamic releasing and inhibitory hormones, peripheral hormones and paracrine growth factors. There are two main theories discussed about pituitary tumor genesis. According to one theory, adenomas arise from a mutational transforming event in a single cell that then will proliferate under the effect of hormones and growth factors, originating a monoclonal tumor. The second theory posits that hypothalamic hormones and growth factors induce pituitary hyperplasia, in which a mutational event in a hyperplastic cell will lead to tumor formation. Overexpressed oncogenes described in pituitary tumorgenesis are: G proteins [34-35], Cyclin D1 [36] and growth factors and their receptors [37-40]. Tumor suppressor genes lost or dysregulated in pituitary adenomas include: MEN-1 encoding the protein menin [41], Rb [42-43], p16 [44], p27 [45], and Zac 15 encoding a zinc finger transcription factor [46].

1.3.2 Cell cycle

Tumor-associated mutations in many of these molecules result in the alteration of the basic regulatory mechanisms that control the mammalian cell cycle. Cell cycle dysregulation can lead to uncontrolled cell proliferation and consequently to tumor development. The basic cell cycle is divided into four phases (Fig. 3). During two of these phases, cells execute the two basic events in cell division: generation of a single and faithful copy of its genetic material (the synthetic or S phase) and partitioning of all the cellular components between two identical daughter cells (mitosis or M phase). The two other phases of the cycle — G1 and G2 — represent 'gap' periods, during which cells prepare themselves for the successful completion of the S and M phases, respectively. When cells halt proliferation, either due to specific antimitogenic signals or to the absence of proper mitogenic signaling, they exit the cycle and enter a non-dividing, quiescent state known as G0.

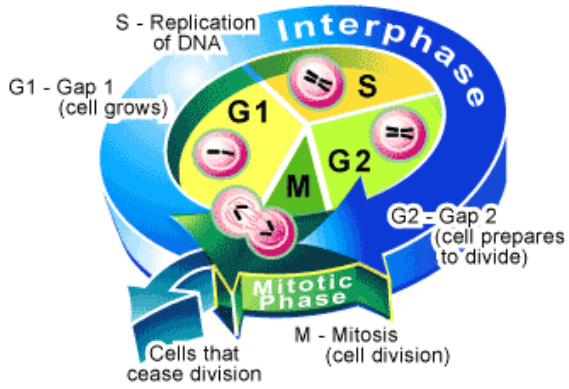


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Fig. 3: The basic cell cycle

To ensure proper progression through the cell cycle, cells have developed a series of checkpoints that prevent them from entering into a new phase until they have successfully completed the previous one [47]. It is obvious that newly divided or quiescent cells must also pass certain checkpoints before they can enter the cycle. For instance, cells must make sure that they have reached their homeostatic size, otherwise cells will become smaller with each round of division. The genetic elements that control

these parameters remain largely unknown. However, they can be influenced by external elements, such as the amount of available nutrients or the intensity of the mitogenic stimulation that cells receive at any given time. Indeed, these two parameters are key determinants for cell division. For instance, if cells have mitogenic requirements that are too stringent, they might not be able to proliferate at crucial times, such as during wound healing or during an infection. Conversely, loosening these controls might lead to unscheduled proliferation and possibly neoplastic growth. In culture, cells undergo a period of mitogen dependence before they enter the cycle. This transition — the Restriction Point (R) — represents a point of no return that commits cells to a new round of cell division [48].

The cyclin-dependent kinases (CDKs), the cyclin-dependent kinase inhibitors (CKIs) and their molecular regulators

Cyclin-dependent kinases are heterodimeric enzymes with a protein kinase subunit and a cyclin subunit that are regulated by various chemical changes. The kinase is completely inactive without its cyclin partner, but in addition to the binding of the cyclin, activation of the holoenzyme requires the phosphorylation of a key residue in the activation loop of the kinase subunit [49]. An overview of the CDKs and its cyclin partners and how they are regulated is given in figures 4 and 5. By binding, the CDK gets phosphorylated and activates or inactivates target proteins to coordinate entry into the next phase.

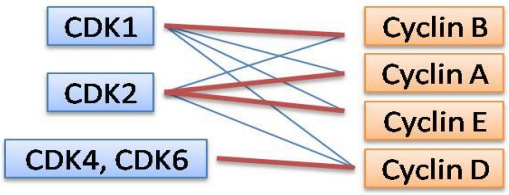


Fig. 4: CDKs and its cyclin partners

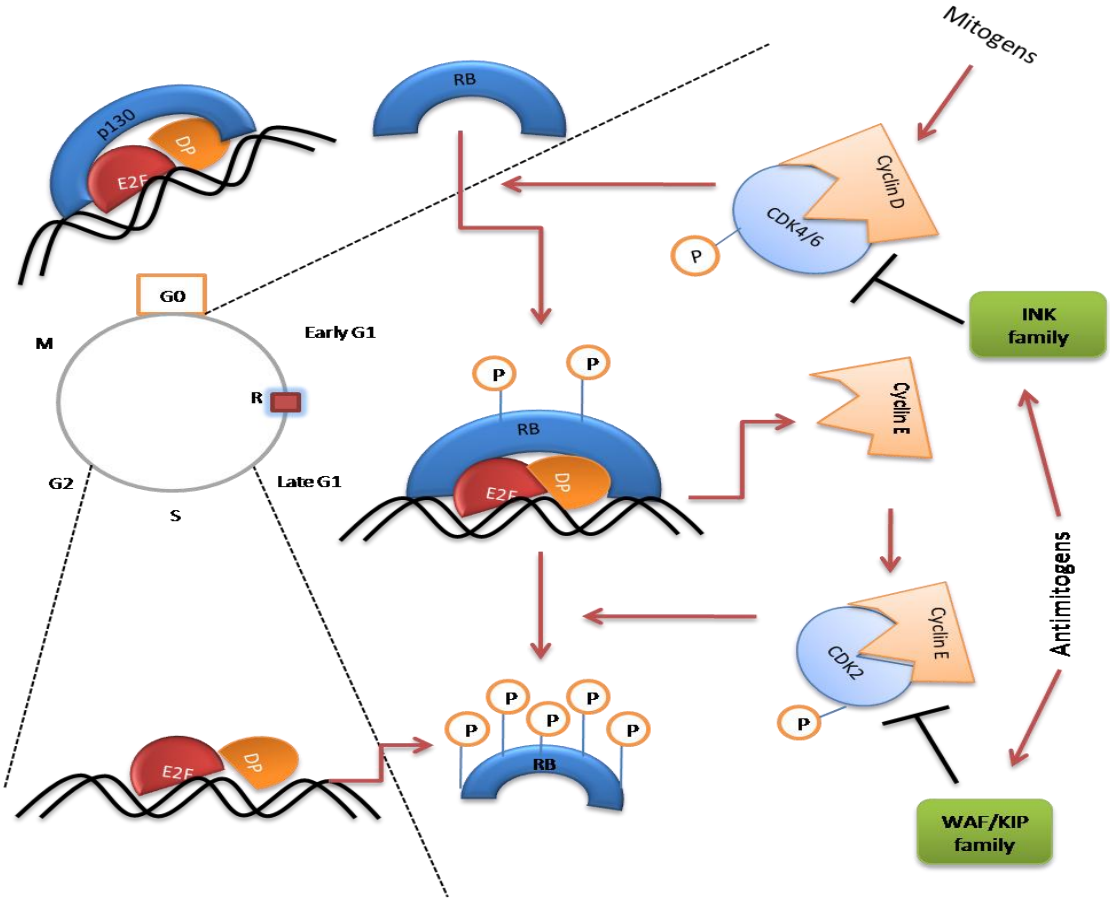


Fig. 5: Regulation of G1 phase and G1/S transition. In quiescent G0 cells, E2F–DP transcription factors are bound to p130, the principal pocket protein in these cells, which keeps them inactive. Mitogenic signaling results in Cyclin D synthesis, formation of active CDK4/6–Cyclin D complexes and initial phosphorylation of Rb. Partially phosphorylated Rb still binds to E2F–DP, but the transcription factor is already able to transcribe some genes, such as Cyclin E, which in turn binds to and activates CDK2. It is generally accepted that CDK2-dependent phosphorylation of Rb results in its complete inactivation, which allows induction of the E2F-responsive genes that are needed to drive cells through the G1/S transition and to initiate DNA replication.

The most important restriction point in mammalian cells is in G1 phase. During the early G1 phase and after receiving a mitogenic signal, the Cyclin D, which is the first cyclin produced in the cell cycle, binds to CDK4 and this complex phosphorylates retinoblastoma protein Rb. Rb activates E2F, which finally will lead to the transcription of various genes like Cyclin E; Cyclin E forms a complex with CDK2 and pushes the cell from G1 to S phase. So, according to the classical view Cyclin E – CDK2 is required to

initiate S phase [50]. The negative control on cell cycle progression is exerted by the cyclin-dependent kinase inhibitors (CKIs), which bind to CDK and inhibit their activity [51]. The members of the INK family (p16, p15, p19) inhibit CDK 4/6 in the early G1 phase [52], whereas members of the WAF/KIP family (p21, p27, p57) inhibit CDK2 activity in the late G1 phase [53].

CDK`s are regulated by phosphorylation on threonin residue for proper catalytic activity [54]. The D-type cyclins are important integrators of mitogenic signaling, as their synthesis is one of the main end points of the MAPK pathway. Cyclin D1 is a rather unstable molecule, which is transported from the nucleus to the cytoplasm where it is targeted for proteolysis. Nuclear export is mediated by GSK-3β (glycogen synthase kinase-3β), a kinase that is inhibited by the PI3K/AKT pathway. So, the Cyclin-D1 availability is controlled by a balance between the MAPK (Cyclin-D1 synthesis) on the one hand, and PI3K/AKT (cyclin-D1 stability) pathways, as well as by GSK-3 β (Cyclin-D1 degradation), on the other. [55-56].

The primary substrates for CDK4/6 and CDK2 in G1 progression are the members of the retinoblastoma protein family Rb, p107 and p130 [57]. These molecules function as docking sites for a series of proteins that must be tightly regulated throughout the cell cycle. For instance, Rb proteins bind to E2F family of transcription factors to ensure that they remain inactive during M and G0 phase. In addition Rb-E2F complexes participate in active repression of some promoters, a mechanism that involves other protein families. The activity of the Rb proteins can be modulated by phosphorylation by CDK4-Cyclin-D and CDK2-Cyclin-E complexes [58]. Hyper-phosphorylated Rb proteins release the molecules that bind to their hypophosphorylated isoforms, allowing them to carry out their specific tasks in cell cycle progression.

1.3.3 How tumor suppressors and their companions affect tumor development

Tumor suppressor genes, such as p53 and PTEN, are genes whose products protect the cell from taking the path to cancer. They suppress malignant transformation by regulating cell cycle progression, ensuring the fidelity of DNA replication and chromosomal segregation, or by inducing apoptosis. These transcription factors are induced by oncogenes, DNA damage and other stress signals.

The role of p53 as a tumor suppressor is generally attributed to its ability to stop the proliferation of precancerous cells by inducing cell cycle arrest or apoptosis. So, it exerts influence on the decision whether the cell could live or has to die [59]. The p53 function is disrupted by mutations in about 50% of all human tumors, and in the other half the p53 protein is inactivated by various cellular antagonists. In fact, alterations in p53 function are the most frequent genetic event found in a broad range of human tumors [60]. p53 activates genes which are crucial for the execution of pathways leading to survival or apoptosis such as the members of the BCL-2 family, which includes both, pro-apoptotic proteins like BAX and anti-apoptotic proteins like BCL-2. In this context p53 is shown to physically interact with pro-apoptotic factors like BAX and inhibits survival signals like BCL-2 [61]. p21 is the most famous p53-induced cell cycle inhibitory gene that can induce G1 and G2 arrest, by inhibiting Cyclin E/CDK2 and Cyclin B/CDK1. When p21 is complexed with CDK2, the cell cannot pass through the next stage of cell division. Furthermore, upon induction, p21 inhibits Cyclin E/CDK2 causing hypophosphorylation of Rb that binds E2F to inhibit E2F-mediated transcription of cell cycle genes [62].

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a tumor suppressor gene and is both a protein and lipid phosphatase [63-65]. It is one of the most commonly lost tumor suppressors in human cancer and controls multiple cellular functions such as cell metabolism, proliferation, cell cycle progression and survival. The phosphatase domain has homology to protein tyrosine phosphatases, dual-specificity phosphatases and to tensin and auxilin. This phosphatase is involved in cell cycle regulation, preventing the cells from growing and dividing too rapidly by negatively regulating the PI3K/AKT signaling pathway [66-68]. The lipid phosphatase activity of PTEN can dephosphorylate the D3 position of PtdIns-3,4-P2 and PtdIns-3,4,5-P3, the lipid products of the PI3K lipid kinase activity [69]. Thus, PTEN antagonizes signaling through the PI3K pathway. Indeed, cells lacking PTEN function exhibit a two fold increase in PtdIns-3,4,5-P3 levels [70-71]. One of the positive regulators of PTEN promoter activity is p53 and one of the negative regulators is NF-κB, which has been shown to inhibit PTEN protein expression in several cancers [72-73].

1.4 Innate immunity and cancer

The innate immune system provides a rapid response to pathogens, and in contrast to the adaptive immune system, is constitutively activated. It does not require the existence of cells with memory. Such an immediate activation of innate immunity relies on the detection of conserved microbial motifs by the host, known as pathogen-associated molecular patterns (PAMPs), a diverse family of molecules including lipopolysaccharide, peptidoglycan, bacterial lipoproteins, flagellin and nucleic acid structures from bacteria and viruses. PAMPs are recognized by specific receptors of the innate immune system. Categorically, three PRRs (pathogen recognition receptors) types are known. Extracellular complement, transmembrane TLRs (toll-like receptors) and intracellular NOD (nucleotide oligomerization domain) proteins. After sensing the presence of a PAMP, host innate immune cells initiate a broad spectrum of defense mechanisms that result in the development of inflammation and host resistance to infection: the recruitment of professional phagocytes, the release of antimicrobial peptides and the establishment of a network of cytokines, chemokines and prostanoids [74]. The main purpose of these effects is to kill invading organisms. However, inadequate elimination, prolonged inflammatory signaling and defects in anti-inflammatory responses can all lead to chronic inflammation and benefit tumor development [75].

It is already known that chronic inflammation is involved in tumor initiation (the process by which normal cells are genetically altered so that they become malignant), promotion (the process by which small clusters of malignant cells are stimulated to grow) and progression (the process by which the growing tumor becomes more aggressive). For instance, da Silva Correia et al. showed in MCF-7 cells a mechanism that links innate immunity and tumor growth [76]. Another paper showed the participation of NOD1 in the regulation of colitis-associated colon tumorigenesis [77]. Moreover, Cruickshank et al. could show the involvement of NOD2 in regulating growth and survival of colorectal carcinoma cell lines [78]. In a previous study, our group showed that the transmembrane TLR4 was found not only in immune but also in non-immune cells including normal pituitary cells, and that their activation affected pituitary tumor pathophysiology and progression [79]. Moreover we could recently show the expression of NOD receptors in folliculostellate TtT/GF cells of the anterior pituitary. The results

obtained in that study showed that TLR4 and NOD receptors act in synergy to activate certain events [80]. During my diploma thesis, which was focused on TtT/GF cells, I started to examine the expression of NOD receptors in pituitary adenoma. Although much attention has been paid in recent years to the interaction between host and pathogen, direct interaction between bacteria and tumor cells has been largely overlooked. Nevertheless, it is still unclear how bacteria interact with tumor cells and how the consequences of such interactions influence tumor progression and immunotherapy.

1.4.1 NOD-like receptor family

In humans, the NLR (NOD-like receptor) family is composed of 22 proteins, and there are at least 33 NLR genes in mice. Although primarily expressed in immune cells including both lymphocytes and APCs (antigen-presenting cells) such as macrophages and dendritic cells, NLRs can also be expressed in non-immune cells, including epithelial and mesothelial cells. This family of proteins is defined by a tripartite structure (Fig. 6) consisting of a variable N-terminal protein-protein interaction domain, defined by a CARD (caspase recruitment domain) domain, a central NOD (nucleotide oligomerization domain) domain, which mediates self-oligomerization that occurs during activation, and LRRs (C-terminal leucine-rich repeats), that detects PAMPs [81].

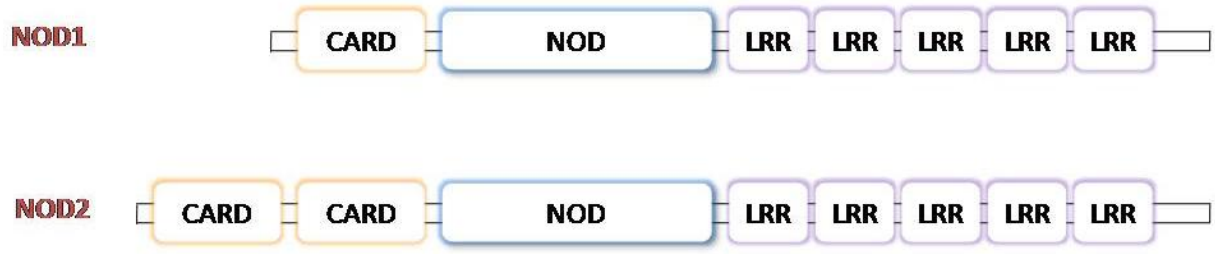


Fig. 6: Structure of NOD proteins. The general structure consists of C-terminal LRRs, a centrally located NOD domain and one or two CARD effector domains.

The N-terminal domain of the NLRs is critical for downstream signaling. CARD domains were originally associated with proteins involved in apoptosis and inflammation such as

many of the caspases, including caspase-1. However, CARDs have also been shown to mediate caspase-independent interactions. CARD possessing proteins are members of the death domain–fold superfamily, members which are involved in both apoptosis and inflammation [82]. Three major activation targets of NOD proteins have been identified: NF-κB, MAPK and Caspase-1 [83-84].

NOD1 (which is encoded by the caspase-recruitment domain 4 gene, CARD4) and NOD2 (which is encoded by CARD15) recognize PGN (peptidoglycan), a component of bacterial cell walls. NOD1 recognizes the dipeptide iE-DAP (γ-D-glutamyl-meso-diaminopimelic) acid, whereas NOD2 recognizes MDP (muramyl dipeptide) [85-86]. As PGN from both, Gram-positive and Gram-negative bacteria contains MDP, NOD2 functions a general sensor of bacteria. Because PGN from Gram-positive bacteria do not contain iE-DAP, NOD1 mainly senses products of Gram-negative bacteria [86-87]. Both NOD1 and NOD2 can be auto-activated upon overexpression. Overexpression of these molecules leads to auto-oligomerization through the NBS (nucleotide-binding site) region [88].

NOD receptor signaling

NOD1 and NOD2 are responsible for the activation of both the NF-κB (nuclear factor kappa B) and the MAPK (mitogen-activated protein kinase) pathways [89]. Upon recognition of their respective agonists, both NOD1 and NOD2 self-oligomerize to recruit and activate the adaptor protein RICK (receptor interacting protein-like interacting caspase-like apoptosis regulatory protein kinase), which is essential for the activation of both NF-κB (Fig. 7) and the MAPKs [90-91]. RICK (also known as RIP2) is a CARD-containing kinase and becomes polyubiquitinated upon interaction with NOD1 or NOD2 through homotypic CARD-CARD interactions. The RICK ubiquitination step is essential for recruitment of the kinase TAK1 (TGFβ-activated protein kinase 1), which activates the NF-κB-activating complex [92].

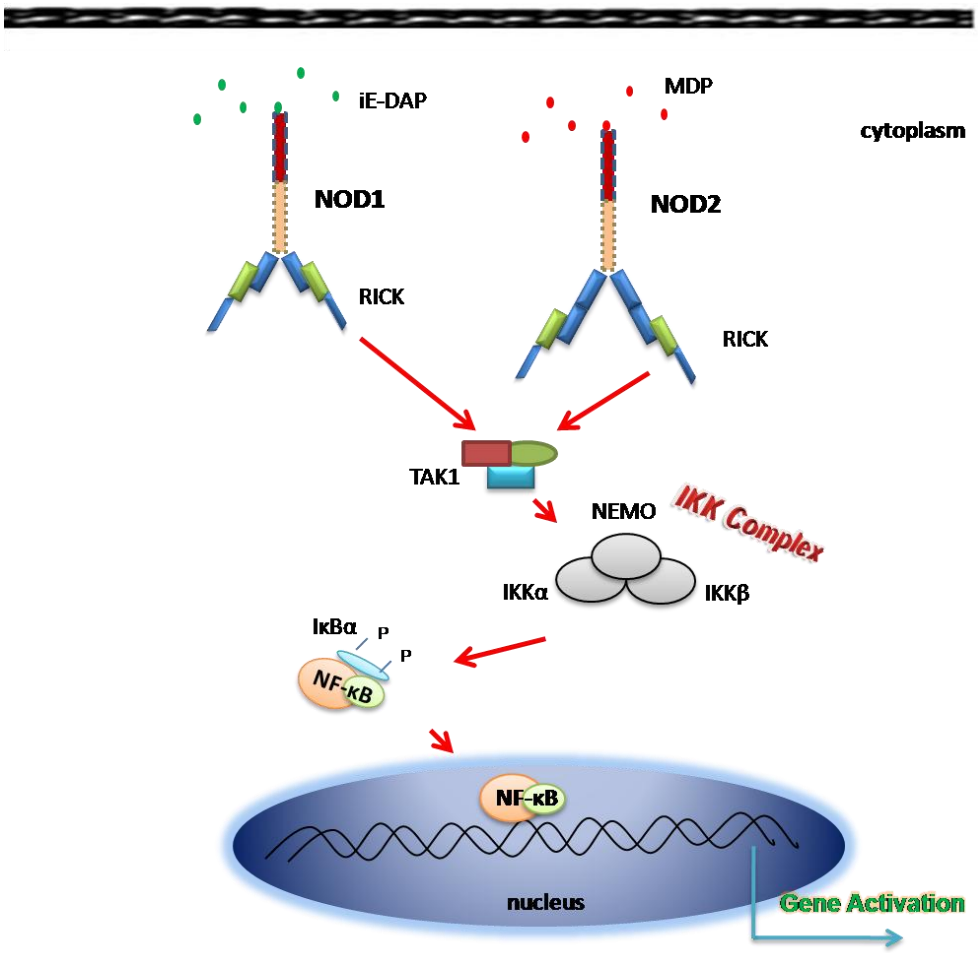


Fig. 7: NOD proteins mediated NF-κB activation. NOD receptor activation through their ligands iE-DAP and MDP recruits the adapter molecule RICK, which in turn recruits the TAK1 kinase. Activation of the IKK complex (NEMO, IKKα and IKKβ) phosphorylates the inhibitory protein IκBα, which leads to its proteosomal degradation; thus, liberating NF-κB dimers and allowing them to regulate gene transcription in the nucleus.

The colocalization of NEMO (NF-κB essential modulator) and TAK1 results in the phosphorylation and degradation of IκBα and finally to the nuclear translocation of NF-κB [93]. Further details on NF-κB activation will be given in chapter 1.5.1. NF-κB subsequently activates transcription of inflammatory cytokines and chemokines such as TNF-α, IL-6 and IL-8, which are important for stimulation and recruitment of additional effector cells during host defense. NF-κB is also involved in proliferation and in the regulation of members of the BCL-2 family, which consist pro-apoptotic and anti-apoptotic proteins.

1.4.2 NOD proteins in diseases and cancers – an overview

Different mutations of NOD2 can lead to decreased or increased NF-κB activity, causing two distinct phenotypes. While gain of function mutations have been associated with BS (Blau Syndrome), other mutations considered to be loss of function have been associated with Crohn`s Disease (CD). The mutation in case of Blau Syndrome is said to be located in the NOD domain, while the mutation causing Crohn`s Disease is located in the LRR domain. Most epithelial cells constitutively express NOD1, whereas NOD2 is restricted to Paneth cells – secretory epithelial cells located at the base of intestinal crypts. They secrete antibacterial substances, initially located in granules within the cytosol, in response to prokaryotic rather than eukaryotic pathogens. The main antimicrobial factors secreted by the Paneth cell include lysozyme, phospholipase A2, trypsin, α-defensins and agiogenins. NOD2 expression is located in close proximity to the secretory granules. Indeed, this close proximity prompts speculation that NOD2 may be involved in degranulation and mediator release. As the mutation in the case of Crohn`s Disease is located in the LRR region, these cells have a decreased NF-κB response to MDP. Therefore, impairing NF-κB response and production of α-defensins, that normally keeps the commensal bacterial population downregulated, leads to overgrowth of the intestinal microflora and increased susceptibility to enteric pathogens, triggering an adaptive immunity hyper-responsiveness that causes and maintains inflammation [94].

Abnormalities in the normal function of NOD1 can lead to development of chronic infection with helicobacter pylori and to the subsequent development of peptic-ulcer disease and gastric carcinoma [95-96]. It has been shown that variation in the NOD1 gene is correlated with the development of asthma, as the NOD1 gene is located in a region which genetically has been linked to asthma [97].

To summarize, NOD proteins are excellent examples of the crosstalk between innate immunity and control of inflammation. Physiologically, NOD proteins are involved in intracellular sensing of bacterial components, but when dysregulated, they lead to inflammatory diseases and to the development of cancers.

1.5 Participating signaling pathways

1.5.1 NF-κB (nuclear factor kappa B)

One transcription factor that serves as a key responder to changes in the cells` environment is NF-κB, an evolutionarily conserved signaling pathway that plays a critical role in many biological processes. The five members of the mammalian NF-κB family, p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2), exist in unstimulated cells as homo- or heterodimers bound to IκB family proteins. NF-κB proteins are characterized by the presence of a conserved 300-amino acid Rel homology domain (RHD), which is located toward the N terminus of the protein and is responsible for dimerization, interaction with IκBs and binding to DNA. The cascade of NF-κB activation by NOD signaling is depicted in figure 8.

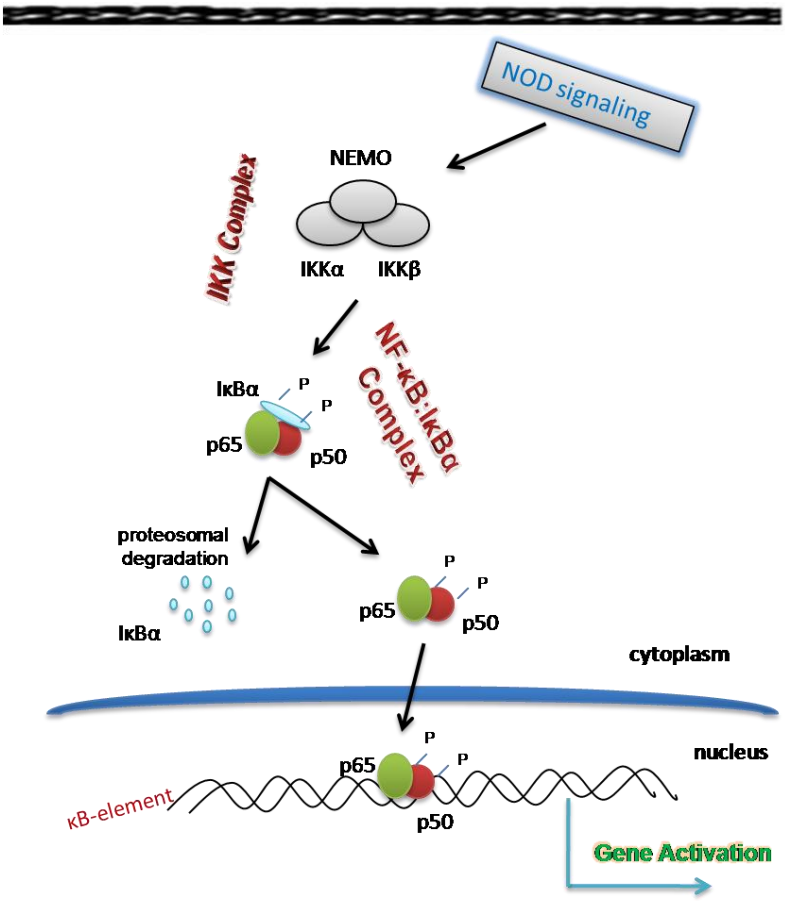


Fig. 8: Schematic representation of the NF-κB pathway activation. NOD signaling activates the IKK complex, which phosphorylates the inhibitory IκBα protein, triggering its subsequent ubiquitination and degradation by the proteasome (IKK Complex). Removal of IκBα enables newly freed NF-κB dimers to undergo post translational modification, such as p65 phosphorylation, and translocate to the nucleus. They bind to their specific DNA element and ultimately activate transcription of target genes.

NF-κB transcription factors stand out as master regulators of innate and adaptive immunity and inflammation [98]. NF-κB also promotes cell survival, and this activity is crucial for antagonism of programmed cell death, tumorigenesis and chemoresistance in cancer. Indeed, whereas NF-κB contributes to many aspects of oncogenesis, including cell proliferation, tissue invasiveness, angiogenesis and metastasis formation, it is now clear that its suppressive action on apoptosis is central to this process [99].

Recent studies have put new light on the basis for this pivotal role of NF-κB in carcinogenesis. One common event in cancer is inflammation, and indeed, chronic infections and inflammation account for 15–20% of all cancer deaths in humans. Notably, it was shown that a crucial link between inflammation and carcinogenesis depends on NF-κB – documented in both, humans and animal models. In this link NF-κB promotes synthesis of inflammatory mediators, such as TNFα, that potently stimulate growth of cancer cells, and upregulates genes that protect these cells against programmed cell death induced by proinflammatory signals [100-101].

1.5.2 MAPKs (mitogen-activated protein kinases)

The MAPKs are one of nature's preferred "solutions" for signaling, and the basic three-kinase module (Fig. 9) has been replicated for various tasks in all eukaryotes.

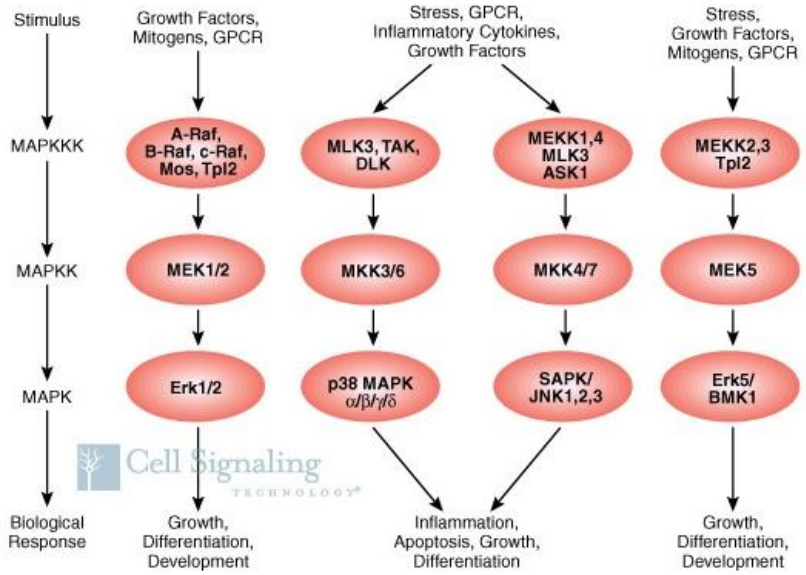


Fig. 9: MAPK modules. These pathways respond cellular signals including growth factors, hormones and cell stress cytokines. Once activated, MAPKs can phosphorylate a wide range of proteins, including transcription factors and other kinases [102].

While the overall structure of the module is remarkably conserved, the inputs and outputs are diverse. MAPKs are now commonly used as examples of an entire class of protein serine kinases that share several key features, including regulation by tyrosine and threonine phosphorylation and organization into a hierarchical cascade of kinases. Upon stimulatory effects the MAPKKKs come into close proximity to the MAPKK and appropriate MAPK, thus leading ultimately to MAPK phosphorylation and activation. Once stimulated the MAPK can then bind and stimulate other kinase targets, translocate to the nucleus to activate gene transcription or induce other actions [103]. These MAPK pathways are essential for processes such as gene expression, mitosis, differentiation, proliferation, cell survival and apoptosis [104]. To date, the sequences of over 100 eukaryotic MAPKs have been reported. In mammals, three major MAPK signaling modules have been described: the original MAPK or ERK (extracellular signal-regulated kinase) and two MAPK cascades that respond to cellular stresses, JNKs (SAPK or c-Jun NH2-terminal kinase) and P38 MAPKs [105].

The ERK (extracellular signal-regulated kinase) Kinase

In mammals, the prototypical MAPKs are encoded by two genes, ERK-1 and -2, generating proteins of 44 kDa and 42 kDa, respectively. Like most of the MAPK proteins, these enzymes are widely expressed and are generally not regulated at the transcriptional level. These enzymes are phosphorylated and activated by MAPK/ERK kinases (MEKs) 1 and 2 [106]. ERK activation is often associated with proliferation, but consequences of ERK stimulation depends on the signal and cell type. Even in the same cell type, the kinetics of ERK activation play a defining role in the ultimate response. Thus, the same signaling machinery can deliver different messages within a cell. The signaling pathways which the members of these families influence, can be independent of each other, or overlapping [107].

JNKs (c-Jun N-terminal kinase)

While the ERK family of MAPKs is primarily activated by growth factors and mitogens, the JNK and P38 MAPK families are preferentially induced by stress signals including irradiation, pro-inflammatory cytokines, environmental stress and other circumstances

important for the cellular environment. The JNKs consist of ten isoforms derived from three genes (JNK2, JNK3 and JNK1), that encode 54 kDa or 46 kDa proteins [108]. The JNKs were originally identified as the major serine/threonine kinases responsible for the phosphorylation of c-Jun, a component of the AP-1 transcription factor [109]. The activated JNKs mostly target transcription factors. The general consequence of chronic JNK signaling is induction of apoptosis.

P38 MAPK

The P38 MAPK group consists of four members: P38α, P38β, P38γ and P38δ. These proteins are encoded by separate genes and are approximately 60% identical at the amino acid level. The P38 MAPKs affect a variety of intracellular responses with well-recognized roles in inflammation, cell death, development, differentiation, senescence and tumorigenesis [110]. Recently it has been shown that P38 is involved in the regulation of cell cycle and the initiation of cell cycle checkpoints to mediate cell survival [111].

1.5.3 PI3K (Phosphoinositide 3-kinases)/AKT pathway

The family of lipid kinases named PI3Ks (phosphoinositide 3-kinases) has been found to have key regulatory roles in many cellular processes including cell survival, proliferation and differentiation [112]. As major effectors downstream of receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs), PI3Ks transduce signals from various growth factors and cytokines into intracellular messages by generating phospholipids, which activate the serine-threonine protein kinase AKT (also known as protein kinase B, PKB) and other downstream effector pathways. PI3Ks are divided into three enzyme classes, according to their structural characteristics and substrate specificity [113]. The most commonly studied ones are the class I enzymes, that are activated directly by cell surface receptors. They are further divided into class IA, which are activated by RTKs, GPCRs and certain oncogenes, and class IB, which are regulated exclusively by GPCRs.

Signaling through this pathway can start from binding of growth factor with its receptor. The activated receptors undergo auto-phosphorylation and acquire tyrosine kinase activity. The activator and the PI3 kinase then activates PI(4,5)P2 into phosphatidylinositol-3,4,5-triphosphate PI(3,4,5)P3, which can be reversed by PTEN,

which acts as an antagonist. PDK-1/2 and AKT are recruited to the plasma membrane by binding to PI (3,4,5) P3 via their pleckstrin homology (PH) domains. After localization to the membrane, PDK-1/2 is able to activate AKT by phosphorylation at Thr 308 and Ser 473. Activated AKT subsequently phosphorylates many downstream proteins. A summary of PI3K/AKT pathway activation and their targets is depicted in figure 10.

The serine-threonine protein kinase AKT mediates many of the downstream effects of PI3K and consequently plays a central role in both normal and pathological signaling by the PI3K pathway. There are three closely related enzymatic isoforms AKT1 (PKBα), AKT2 (PKBβ) and AKT3 (PKBγ), encoded by three different genes located on chromosomes 14q32, 19q13 and 1q43, respectively. They are similar in both, structure and size, and are thought to be activated by a common mechanism [114]. AKT has additional effects on tumor-induced angiogenesis that is mediated, in part, through HIF-1α (hypoxia-inducible factor-1α) and VEGF (vascular endothelial growth factor). The main biological consequences of AKT activation, which are relevant to cancer-cell growth, can be catalogued loosely into three categories — survival, proliferation (increased cell number) and growth (increased cell size). These three categories will be discussed more extensively in the following paragraphs and are also depicted in figure 10.

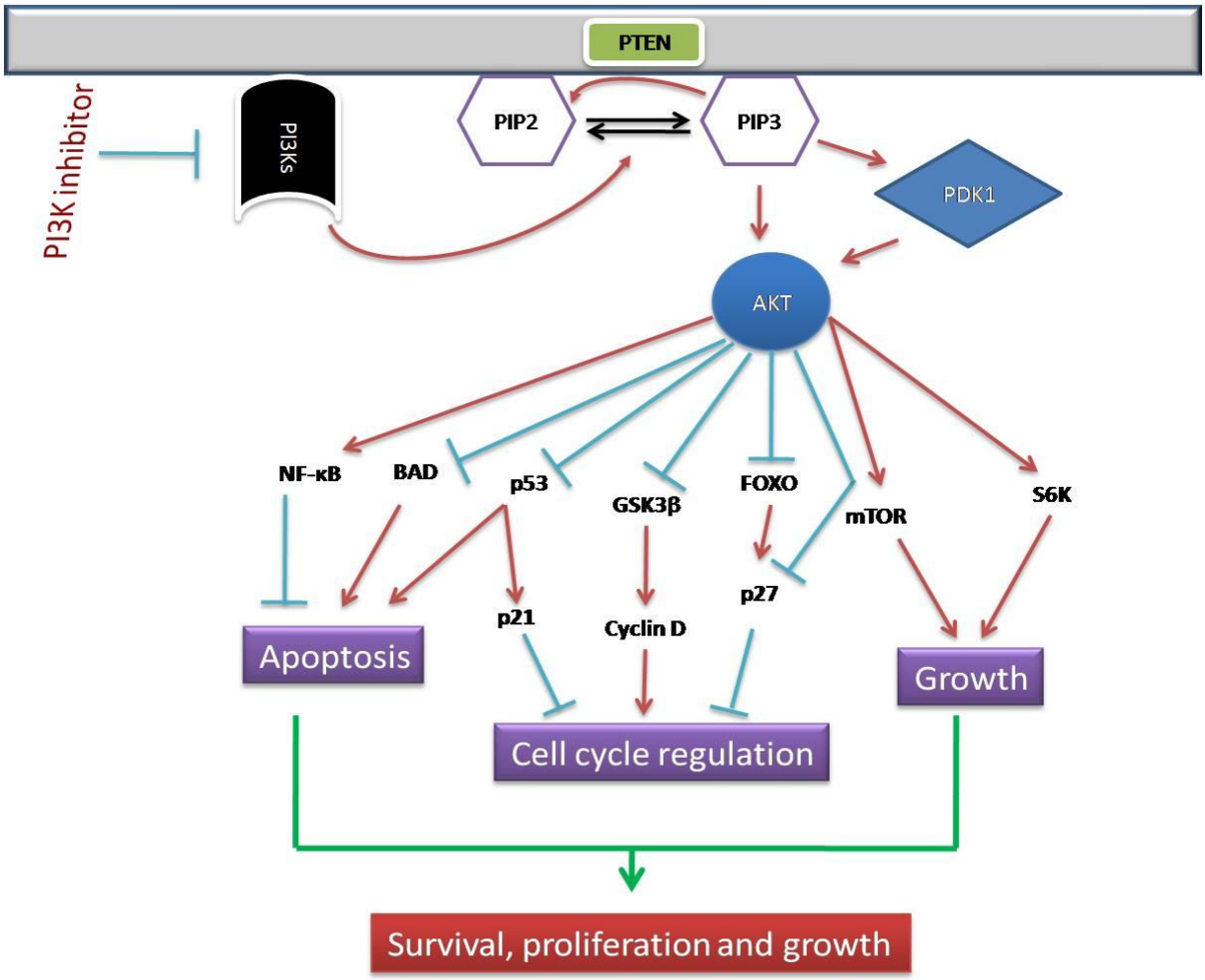


Fig. 10: PI3K/AKT pathway and its influence on downstream proteins

Survival versus Apoptosis

Apoptosis, or programmed cell death, is a normal cellular function that controls excessive proliferation by eliminating 'unnecessary' cells. Cancer cells have devised several mechanisms to inhibit apoptosis and prolong their survival. AKT functions in an anti-apoptotic manner. Among the many substrates of AKT, BAD proteins dissociate from BCL-2 or BCL-XL and lose the ability to induce apoptosis [115]. AKT can also influence cell survival by means of indirect effects on two central regulators of cell death, the transcription factor NF-κB and p53. AKT can exert a positive effect on NF-κB function by phosphorylation and activation of IKK (IκB kinase), the kinase that induces degradation of the NF-κB inhibitor, IκB, and therefore allowing nuclear translocation

and activation of target genes [116]. Furthermore, AKT can also influence the activity of the pro-apoptotic tumor suppressor p53 through phosphorylation of the p53-binding protein MDM2. MDM2 is a negative regulator of p53 function that targets p53 for degradation by the proteasome. Two recent studies provide a new mode of MDM2 regulation through phosphorylation by AKT. Phosphorylated MDM2 translocates more efficiently to the nucleus, where it can bind p53, resulting in enhanced p53 degradation [117]. AKT is also known to inactivate FOXO, which regulates transcription of pro-apoptotic proteins such as Fas-ligand (FasL) and Bim [112].

Proliferation

AKT also controls cell cycle progression by inactivating p27 and GSK-3ß (glycogen synthase kinase-3ß). p27, a cyclin-dependent kinase inhibitor, and GSK-3ß play roles in the induction of cell cycle arrest. Phosphorylated p27 is translocated from the nucleus, the site of its activity, to the cytoplasm where it is degraded. In case of GSK-3ß, phosphorylation causes the loss of its kinase activity, thereby supporting the cellular growth response to growth factor stimulation. AKT promotes the G1-S phase transition by blocking FOXO mediated transcription of cell cycle inhibitors, including p27, or directly phosphorylates and inactivates p27, as mentioned before [112].

Cell growth

In addition to its role in proliferation and survival, there is evidence that AKT also affects cell growth. Proliferation refers to cell division, which leads to an increase in cell number, whereas growth refers to the synthesis of macromolecules, which results in increased cell mass or size, a process that is enhanced in cancer cells. One protein that is a central regulator of cell growth is mTOR (the mammalian target of rapamycin,), a serine/threonine kinase that serves as a molecular sensor, which regulates protein synthesis on the basis of the availability of nutrients. mTOR regulates biogenesis by activating p70S6 kinase [118].

There is already evidence that at least NOD2 signaling has an impact on the PI3K/AKT pathway and its components [119]. A lot of evidence has implicated aberrations in the PI3K/AKT pathway in tumorigenesis. The signaling elements of this pathway are found

to be highly activated and dysregulated due to overexpression, truncation or mutation in certain cancer types. Alterations in the PI3K/AKT pathway can lead to abnormal cell functions such as proliferation, differentiation, survival and migration. The accumulation of such uncontrolled cell signaling facilitates the formation of cancer [120].

2. Aim of the study

The previous chapters presented some aspects of NOD signaling and its potential involvement in the regulation of cell survival, proliferation, cell growth and invasion. Nevertheless, the understanding of the network of interactions between the various factors that determine proliferation, survival or death, remains limited. Proteins which, up to now, have not been recognized to play a role in this machinery may eventually prove to be missing links in the pathways involved.

There is a large body of evidence suggesting that innate immune receptors activation plays a role in tumor growth and development. To better understand how NOD proteins are involved in regulating proliferation, survival and apoptosis, it is particularly important to know more about the properties of these new intracellular proteins NOD1 and NOD2. In this context, the main goal of this dissertation was to determine the expression and effects of innate immune receptors NOD1/2 signaling pathways in endocrine pituitary cells and pituitary adenomas.

3. Materials & Methods

3.1 Materials

3.1.1 Equipment

Analytic Balance 1601 MP8	Sartorius AG (Göttingen, Germany)
Cell culture bottles	Nunc (Denmark)
Cell culture incubator Cytoperm 8080	Heraeus GmbH (Hanau, Germany)
Cell culture plates	Nunc (Denmark)
Cell scraper	Renner (Dennstadt, Germany)
Centrifuge Sepatech Varifuge 3	Haraeus GmBH (Osterode, Germany)
Centrifuge Tubes	Falcon (Heidelberg, Germany)
ECL Western Blotting Detection System	Amersham Biosciences (Uppsala, Sweden)
ELISA – plate reader Dynatech MR 5000	Dynatech (Denkendorf, Germany)
Phase Lock Gel Heavy Tubes	5 Prime GmbH (Hamburg, Germany)
Laminar Flow Typ UVF 6.18 S	BDK Luft und Reinraumtechnik GmbH (Sonnenbühl, Germany)
TriStar LB 941	Berthold Technologies (Tennessee, USA)
Microscope Axiskop 2	Carl Zeiss AG (Jena, Germany)
Neubauer counting chamber, Blaubrand®	BRAND GMBH + CO KG (Wertheim, Germany)
Novex® Semi Dry Blotter	Invitrogen Corp. (Paisley, UK)
Pipettes	Eppendorf (Hamburg, Germany)
PVDF (Polyvinylidene fluoride) membranes	Millipore Corp. (Bedford, MA, USA)

Shaker Scientific Industries Vortex-Genie®-2	Scientific Industries Inc. (New York, USA)
Spectrophotometer Beckman DU-640	Beckman Instruments Inc. (Miami, FL, USA)
Eppendorf Table Centrifuge 5415R	Eppendorf (Hamburg, Germany)
Thermocycler- Techgene	Labtech International (East Sussex, UK)
Tris-Glycine 10% gel	Anamed (Groß-Bieberau, Germany)
Water bath	Köttermann Labortechnik (Uetze-Hänigsen, Germany)
XCell SureLock™ Mini-Cell	Invitrogen Corp. (Paisley, UK)

3.1.2 Reagents

ABC kit	Vector Laboratories (Burlingane, CA, USA)
Acridine orange	Sigma (St. Louis, MO, USA)
BSA (Bovine serum albumin)	Sigma (St. Louis, MO, USA)
Cell lysis buffer	Promega Corp. (Madison, USA)
Chloroform	Sigma (St. Louis, MO, USA)
Collagenase	Worthington Biochemical Corp. (Lakewood, NJ, USA)
Developer Solution	Kodak (Stuttgart, Germany)
Diaminobenzidine (DAB)	Sigma (St. Louis, MO, USA)
Diethyl-pyrocarbonate (DEPC)	Sigma (St. Louis, MO, USA)
Dimethylsulfoxid (DMSO)	Sigma (St. Louis, MO, USA)

Dithiothreitol (DTT)	Sigma (St. Louis, MO, USA)
DNAse I	Invitrogen Corp. (Paisley, UK)
dNTP Mix	MBI Fermentas (Vilnius, Lithouania)
Dulbecco`s modified Eagle medium (DMEM)	Invitrogen Corp. (Paisley, UK)
Ethanol	Merck (Darmstadt, Germany)
Ethylenediaminotetracetic acid (EDTA)	Merck (Darmstadt, Germany)
Ethidium bromide	Sigma (St. Louis, MO, USA)
Fetal Calf Serum (FCS)	Gibco (Karlsruhe, Germany)
Fixer solution	Kodak (Stuttgart, Germany)
3H-Thymidine	Amersham Biosciences (Uppsala, Sweden)
Hexanucleotide Mix	Roche (Mannheim, Germany)
iE-DAP (γ-D-glutamyl-meso diaminopimelic)	Invivogen (San Diego, California, USA)
Isopropanol	Sigma (St. Louis, MO, USA)
L-Glutamine	Biochrome AG (Berlin, Germany)
Lipofectamine 2000	Invitrogen Corp. (Carlsbad, CA, USA)
Luciferin Detection Reagent	Promega Corp. (Madison, USA)
Lumi-Light Western Blotting Substrate	Roche (Mannheim, Germany)
LY294002 (PI3 Kinase Inhibitor)	Calbiochem (La Jolla, CA, USA)
Marker Precision Plus Protein™Standards	Bio-Rad (California, USA)
1kb Plus DNA Ladder™	Invitrogen Corp. (Paisley, UK)

MDP (Muramyl dipeptide)	Bachem AG (Bubendorf, Switzerland)
Methanol	Merck (Darmstadt, Germany)
Milk powder (Frema Reform Instant Magermilchpulver)	GRANOVITA GmbH (Heimertingen, Germany)
ONPG solution	Sigma (St. Louis, Mo, USA)
Optimem medium	Gibco Europe (Karlsruhe, Germany)
Paraformaldehyde (PFA)	Merck (Darmstadt, Germany)
Partricine	Biochrome AG (Berlin, Germany)
Phosphate based buffer (PBS)	Gibco Europe (Karlsruhe, Germany)
PhosST*O*P Phosphatase Inhibitor Cocktail Tablets	Roche (Mannheim, Germany)
Penicillin + Streptavidine mix	Biochrom AG (Berlin, Germany)
Phenol	Roth (Karlsruhe, Germany)
Polyacrylamide	Invitrogen Corp. (Paisley, UK)
Ponceau S Solution	Sigma (St. Louis, Mo, USA)
Protease Inhibitor Cocktail	Sigma (St. Louis, Mo, USA)
RNAse A	Roche (Mannheim, Germany)
RNAsin (RNAase inhibitor)	Promega Corp. (Madison, WI, USA)
Roti-Histokitt	Roth (Karlsruhe, Germany)
Roti®-Load1	Roth (Karlsruhe, Germany)
Reverse transcriptase (SuperScript II TM)	Invitrogen (Carlsbad, CA, USA)
Running buffer (SDS)	Anamed (Groß-Bieberau, Germany)

Sodium acetate dihydrate	Merck (Darmstadt, Germany)
Sodium acetate trihydrate	Merck (Darmstadt, Germany)
Sodium chloride (NaCl)	Roth (Karlsruhe, Germany)
Sodium citrate dihydrate	Merck (Darmstadt, Germany)
Sodium dihydrogen phosphate monohydrate (NaH_2PO_4-H_2O)	Merck (Darmstadt, Germany)
Sodium hydrogen phosphate dihydrate (Na_2HPO_4-$2H_2O$)	Merck (Darmstadt, Germany)
Sodium peroxyde (NaOH)	Merck (Darmstadt, Germany)
Streptavidine-horseradish-Peroxidase-complex	R&D Systems (Wiesbaden-Nordenstadt, Germany)
Taq DNA polymerase	MBI Fermentas
Toluidine Blue O	Sigma (St. Louis, Mo, USA)
Transferrin	Sigma (St. Louis, Mo, USA)
Trichloroacetic acid	Roth (Karlsruhe, Germany)
Tris pure	ICN Pharmaceuticals (Aurora, OH, USA)
Triton X-100	Roth (Karlsruhe, Germany)
TRIZOL	Invitrogen (Carlsbad, CA, USA)
Trypsin	Sigma (St. Louis, Mo, USA)
Tween 20	Sigma (St. Louis, Mo, USA)
Ultima Gold Scintillation Solution	Packard Bioscience (Gromingen, Netherlands)

3.1.3 Solutions

Collagenase Mix	1000 U/ml Collagenase: 4 g/ 100 ml solution Trypsin inhibitor: 10 mg/ 100 ml solution Hyaluronidase: 100 mg/ 100 ml solution BSA: 400 mg/ 100 ml solution Dnase: 500 µl/ 100 ml solution
HDB+ buffer	Hepes: 5.95 g/l NaCl : 8 g/l KCl: 0.37 g/l Na_2HPO_4-H_2O: 0.12 g/l Glucose: 1.982 g/l Partricine 25 µg/ml: 10 ml Penicillin/Streptomycin 10^5 U/l : 10 ml Adjust pH to 7.3 with NaOH Store at +4°C
ONPG buffer 2x	1 M Na_2HPO_4 44 ml 1 M NaH_2PO_4 16.13 ml H_2O 270 ml $MgCl_2$-$6H_2O$ 123 mg ONPG 398 mg stirring 40 minutes

	+ 2 ml B-Mercaptoethanol (14M)
Stripping buffer	to prepare 500 ml solution: 50 ml 20% SDS 25 ml 1 M Tris/HCl pH 7.4 425 ml H_2O + add DTT 50 mM (final concentration)
RIPA buffer	50 mM Tris HCl pH 8 150 mM NaCl 1% NP-40 0.5% Sodium Deoxycholate 0.1% SDS
Running buffer 1x	to prepare 1 l solution: anamed running buffer: 100 ml + 900 ml distilled water
Paraformaldehyde (PFA) 4%	paraformaldehyde: 4 g/100 ml Sodium phosphate buffer: 20 ml/100 ml Ampuwa water: 80 ml Add 1 M NaOH to pH 7.4 Heat at 56°C to dissolve Filter and cool before usage Store at +4°C for maximum 2 days
Transferbuffer	14.4 g Glycin

	3.03 g Tris-Base 0.5 g SDS 200 ml Methanol completed with 800 ml H_2O
Tris borate EDTA buffer (TBE) 10x	Boric acid (H3BO3): 61.83 g/l EDTA: 37.2 g/l Tris pure: 30.03 g/l pH: 8.0
Tris buffer (TB)	Tris pure: 12.114 g/l pH: 7.6
Tris-based buffer (TBS)1x	Tris pure: 2.42 g/l NaCl: 8 g/l pH: 7.6
TBST	TBS + 0.1% Tween 20
Tumor medium	DMEM (Dulbecco´s Modified Eagle´s Medium) 2 mM Essential Vitamins 40 U/l Insulin 20 ng/l Natrium Selenate 5 mg/l Transferrin 30 pM Triiodothyronine (T_3) 10% Fetal Calf Serum

	2 mmol/l L-glutamine 2.5 ng/l Partricine 10^5 U/ml Penicillin-Streptomycin

3.1.4 Antibodies

Primary antibodies used for Western immunoblotting

Target protein	Species; dilution	Company
BAX	rabbit; 1:1000	Cell Signaling Technology
BCL-2	rabbit; 1:1000	Cell Signaling Technology
CDK2	rabbit; 1:1000	Cell Signaling Technology
CDK4	mouse; 1:1000	Cell Signaling Technology
Cyclin D1	mouse; 1:1000	Cell Signaling Technology
Cyclin D3	mouse; 1:1000	Cell Signaling Technology
Cyclin E	mouse; 1:1000	Cell Signaling Technology
p21	mouse; 1:500	Cell Signaling Technology
p-AKT Ser473	rabbit; 1:2000	Cell Signaling Technology
p-AKT Thr308	rabbit; 1:2000	Cell Signaling Technology
p-ERK1/2	rabbit; 1:1000	Cell Signaling Technology
p-FOXO	rabbit; 1:1000	Cell Signaling Technology
p-GSK-3β	rabbit; 1:1000	Cell Signaling Technology
p-JNK	mouse; 1:1000	Santa Cruz Biotechnology
p-mTOR Ser2448	rabbit; 1:1000	Cell Signaling Technology

p-NF-κB	rabbit; 1:1000	Santa Cruz Biotechnology
p-P38	mouse; 1:1000	Santa Cruz Biotechnology
p-p70S6K Thr389	rabbit; 1:1000	Cell Signaling Technology
p-PTEN	rabbit; 1:1000	Cell Signaling Technology
p-Raptor Ser792	rabbit; 1:1000	Cell Signaling Technology
p-Rb Ser780	rabbit; 1:500	Cell Signaling Technology
p-Rb Ser795	rabbit; 1:500	Cell Signaling Technology
t-AKT	rabbit; 1:1000	Cell Signaling Technology
t-ERK 1/2	rabbit; 1:1000	Cell Signaling Technology
XIAP	rabbit; 1:1000	Cell Signaling Technology
β-actin (beta-actin)	mouse; 1:20.000	Chemicon

Secondary antibodies used for Western immunoblotting

Name	Dilution	Company
anti-rabbit-HRP	1:1000	Amersham Biosciences (Freiburg, Germany)
anti-mouse-HRP	1:1000	Amersham Biosciences (Freiburg, Germany)

Antibodies used for Immunohistochemistry

Antigens	Primary antibodies	Secondary biotinylated antibodies
NOD1 (E-14)	Goat anti-human (Santa Cruz Biotechnology, Santa Cruz, CA, USA)	Horse anti-goat (Vector Laboratories Inc., Burlingame, CA)
NOD2 (P-18)	Goat anti-human (Santa Cruz Biotechnology, Santa Cruz, CA, USA)	Horse anti-goat (Vector Laboratories Inc., Burlingame, CA)

3.2 Human tissues

This study was performed after approval of the Max Planck Institute`s ethics committee (ethics appliciation number: 141-07) and informed consent was received from each patient or their relatives. 4 normal human pituitaries and 33 pituitary adenomas were included in the study. The pituitary tumors were obtained from the neurosurgical clinic (LMU Klinikum Großhadern). The normal pituitary glands (NP) were obtained from the autopsy of the Institute of Forensic Medicine less than 20 hours post mortem. The studied pituitary tumors were obtained from transsphenoidal surgery of 33 patients: 15 males and 18 females, classified according to clinical presentation into 7 somatotropinomas (ACRO), 6 corticotropinomas (CUSH), 8 non functioning pituitary adenomas (NFPA), 8 prolactinomas (PROL) and 4 thyreotropinomas (TSH) (Table 1). All the tumors were benign and tumor grade was determined according to a modified Hardy´s classification [121]. Tissue fragments of normal pituitaries and pituitary adenomas were placed in DMEM medium and transferred to our laboratory on ice within 36 hours. After washing, one aliquot was shock-frozen on dry ice and stored at -80°C until use.

Table 1: studied pituitary tumors.

CUSH = corticotropinomas;

NFPA = non functioning pituitary adenomas;

ACRO = somatotropinomas;

TSH = thyreotropinomas;

PROL = prolactinomas;

NP = normal pituitaries

	age	(m/f)	Type		age	(m/f)	type
1	63	f	CUSH	22	40	F	TSH
2	70	f	CUSH	23	58	F	TSH
3	39	m	Nelson	24	40	F	TSH
4	31	f	CUSH	25	55	M	TSH
5	66	f	CUSH	26	43	M	PROL
6	48	f	CUSH	27	34	M	PROL
7	68	m	NFPA	28	66	M	PROL
8	69	f	NFPA	29	34	F	PROL
9	48	f	NFPA	30	37	M	PROL
10	41	m	NFPA	31	46	M	PROL
11	78	f	NFPA	32	79	M	PROL
12	38	f	NFPA	33	28	M	PROL
13	62	m	NFPA	34	-	-	NP
14	74	f	NFPA	35	-	-	NP
15	36	m	ACRO	36	-	-	NP
16	82	f	ACRO	37	-	-	NP
17	56	m	ACRO				
18	28	f	ACRO				
19	42	f	ACRO				
20	38	m	ACRO				
21	84	f	ACRO				

3.3 Cell culture

3.3.1 Primary rat pituitary cell culture and human pituitary cell culture

The pituitary primary cell culture was obtained from adult male Sprague-Dawley rats (180-250 g). They were kept for 5 days in our animal house under standard conditions: 12 hours light/dark rhythm, temperature 21°C, water and standard food. After CO_2 narcosis and decapitation, the pituitary glands were quickly removed and stored in HDB+ buffer. Pieces of human pituitary adenomas, destined for cell culture, were handled in the same way as rat pituitary cell culture cells. Sliced fragments were

enzymatically dispersed in the Collagenase mix buffer at 37°C for approximately 45 minutes. Dispersed cells were centrifuged and resuspended in tumor medium. Cell viability was determined by fluorescence microscopy after staining with acridin orange and ethidium bromide. Acridin orange enters the membranes of normal cells, yielding green fluorescence in viable cells. Ethidium bromide does not pass the healthy cell membrane and enters only in dead cells with damaged membranes, yielding a red fluorescence. Cell viability of pituitary cells was determined as the percentage of green cells in the total number of cells (counted in a Neubauer chamber) and was over 95%. Cells were distributed in 48-well plates (1 x 10^5 cells per well) and incubated at 37°C under 5% CO_2. The stimulation experiments were performed 48 hours after preparation [122].

3.3.2 Immortalized corticotroph cell line AtT20

The expression and functional studies on cell growth and signaling of NOD1 and NOD2 proteins were performed in AtT20 cells. This immortalized corticotroph cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The AtT20 cell line originates from a murine ACTH-secreting pituitary tumor [2]. The medium used for their culture was DMEM supplemented with 10% FCS (fetal calf serum), 1% L-Glutamin, 1% Patricine and 1% Penicillin/Streptomycin. They were kept in 75 cm^2 cell culture bottles in incubators at 37°C in the presence of 5% CO_2. When confluent, the cells were trypsinized, centrifuged at 1200g for 4 minutes and re-dispersed in fresh medium. They were numbered and replaced in new cell culture bottles, or plated in different well-plates according to the planned experiments. For transfections, the cells were plated in 24-well plates at a density of 2 x 10^5 cells per well and all experiments were carried out in triplicate. For hormone measurements and cell proliferation experiments, the cells were plated in 96-well plates using 8 x 10^3 cells per well and in 48-well plates with 1 x 10^5 cells per well, and ultimately carried out in quadruplicate. The experiments were performed 24 hours after cell seeding.

3.3.3 Cell proliferation assay WST-1 and [3H]-thymidine incorporation

24 hours after seeding, the cells were first serum deprived by overnight incubation with serum-free medium before performing the experiment. This step synchronized all the

cells to the cell cycle phase G0. The stimulation with 30 µg/ml of iE-DAP and 30 µg/ml of MDP was stopped after 48 hours.

WST-1 is one of the assays available for analyzing the number of viable cells by measuring the cleavage of tetrazolium salts added to the culture medium. The WST-1 measures the metabolic activity based on the reduction of WST-1 by viable cells. The WST-1 (tetrazolium salt) added to the cells is converted by the mitochondrial dehydrogenase into a dye (formazan), that can be quantified by a multiwall scanning spectrophotometer (ELISA plate reader). The stimulation time was stopped by adding 10 µl WST-1 reagent to each well containing 100 µl of cells in medium. After 15 to 30 minutes, the results were measured in the ELISA reader at 450 nm.

Proliferation was also measured by using the [3H]-thymidine incorporation method. The radioactivity, incorporated in the DNA of the stimulated cells during S phase of the cell cycle, is an indicator of the cells expansion rate. During the last three hours of incubation, 0.5 µCi/ml [3H]thymidine was added, then medium was removed and after one wash in cold PBS, cells were precipitated with ice-cold 10% trichloroacetic acid (1 hour, 4°C) and washed with cold PBS. Then, DNA was hydrolyzed overnight with 0.5 M NaOH/0.1% Triton X-100. The scintillation liquid cocktail, Ultima Gold, was added and the samples were measured in a liquid scintillation counter.

3.4 Molecular Biological Methods

3.4.1 RNA Extraction

RNA was isolated from normal human pituitaries and from human pituitary adenomas using the guanidium isothiocyanate protocol. The tissue piece was first homogenized in 80 µl of solution D containing β–mercaptoethanol, using the Ultra-TURRAX T8 (IKA Labortechnic) tissue homogenizer. Guanidium isothiocyanate and β–mercaptoethanol inhibit the RNAase action activated by cell disruption, preventing in this way RNA degradation. 80 µl of sodium acetate 2 M pH 4.0 was added afterwards, followed by 800 µl of saturated phenol and 160 µl of a chloroform-isoamyl alcohol (49:1) solution. After 15 minutes incubation on ice the samples were centrifuged at 13000 rpm for 20 minutes at 4°C; this step led to the formation of two phases, the upper one containing RNA and the lower one containing DNA and proteins. The upper phase was then transferred to a new tube together with the same volume of ice-cold isopropanol. Incubation of the

sample at –20°C at least for 2 hours was necessary for RNA precipitation. After centrifugation of the sample at 13000 rpm for 10 minutes at 4°C, the supernatant was discarded and the pellet was washed with 70% ice-cold ethanol. After 10 minutes centrifugation at 13000 rpm, the supernatant was again discarded and the pellet was left to dry at room temperature and then dissolved in an appropriate amount of DEPC treated water.

RNA extraction from AtT20 cells was performed as follows: Cells were seeded in 6-well plates, starved overnight and stimulated with 30 μg/ml of iE-DAP and 30 μg/ml of MDP for 24 hours, after which the medium was removed from the wells. 1 ml of TRIzol reagent was added to each well and the cells were passed several times through a pipette to obtain homogenization. During this step, the TRIzol Reagent maintains the integrity of RNA while disrupting cells and dissolving cell components. The homogenized samples were transferred into GEL-tubes (Phase Lock Gel Heavy Tubes, 5 Prime GmbH) and left at room temperature for 5 minutes to allow the complete dissociation of nucleoprotein complexes. Following incubation, 0.2 ml of chloroform was added to the tubes. They were shaken by hand and left again at room temperature for incubation for 2-3 minutes. Afterwards, the tubes were centrifuged at 12000g for 15 minutes at 4°C. Addition of chloroform and centrifugation separates the mixture into a lower phenol-chloroform phase (contains proteins), an interphase (contains DNA) and a colorless upper aqueous phase. The RNA remains exclusively in the aqueous phase. This RNA containing aqueous phase was transferred to fresh tubes and the RNA was precipitated by adding 0.5 ml of isopropyl alcohol to the tubes. Incubation at room temperature for 10 minutes and another centrifugation step followed. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. The supernatant was removed and the RNA pellet washed with 1 ml 75% ethanol. The samples were mixed by vortexing, followed by a final centrifugation step at 7500g for 5 minutes at 4°C. The final step of the procedure was to briefly dry the RNA pellet at 60°C. It is important not to let the RNA pellet dry completely, as this will greatly decrease its solubility. The RNA was dissolved in DEPC water by passing a few times through a pipette tip and incubating for 10 minutes at 60°C.

The samples` absorbance was measured with a photometer and RNA concentration calculated according to the following formula:

$$\text{cRNA} = \frac{\text{A260} * 40 * 60}{1000} \ [\mu\text{g/ml}]$$

A260 is the sample absorbance at 260 nm, 40 is the concentration in µg/µl of RNA giving A260 value equal to one and 60 is the dilution factor used to measure the sample concentration (1 µl RNA+ 59 µl DEPC water). Partially dissolved RNA samples have an A260/A280 ratio < 1.6. The lack of DNA contamination was assessed by performing a PCR reaction for a housekeeping gene like HPRT or β−actin, using the RNA as a sample: if no DNA contamination is present, no band is visible after loading the PCR product on an ethidium bromide gel (as described below).

3.4.2 RT-PCR (Reverse Transcriptase- Polymerase Chain Reaction)

Reverse Transcription is a process in which single-stranded mRNA is reverse transcribed into complementary DNA (cDNA) by using total cellular RNA. The resulting cDNA can be used in PCR reaction. Reverse transcription of RNA samples was performed by incubating the following substances for one hour at 45°C: 1 µg RNA with 1 µl of dNTP mix 2 mM, 2 µl of 62.5 U/ml random primers (Hexanucleotide mix), 2 µl of dithiothreitol (DTT) 10 mM and 200 U reverse transcriptase (SuperScript II), all diluted in 4 µl of 1x first strand buffer and DEPC-water to get a final volume of 20 µl. Reaction was stopped by boiling the samples at 95°C for 5 minutes, followed by cooling down on ice for 5 minutes.

PCR (Polymerase chain reaction) is a rapid method of copying and amplifying specific DNA sequences and is widely used as a preparative and analytical technique. The method relies on the use of short oligonucleotide primers complementary to the sequences at the ends of the DNA to be amplified, and involves repetitive cycles of melting, annealing and synthesis of DNA. One µl of cDNA samples obtained was used for PCR reaction and incubated with 1.5 µl PCR buffer 10x, 0.9 µl $MgCl_2$ 25 mM, 1.5 µl dNTP mix 2mM, 0.5 µl amplification primer sense 10 pmol/µl, 0.5 µl amplification primer anti-sense 10 pmol/µl, 0.15 µl Thermus aquaticus (Taq) DNA polymerase and 8.95 µl

autoclaved distilled water. The PCR reaction consisted of 35 cycles, each containing the following steps: denaturation at 94° C for 1 minute, annealing at 55°C- 65°C according to the employed primers specific annealing temperature (Table 2) for 1 minute and finally elongation of the PCR fragment at 72° C for 1 minute.

Primer	Sequence [5`→ 3`]	Temperature [ºC]	Length [bp]
NOD1 mouse	GTC CTC AAC GAG CAT GGC GAG ACT sense AGC TCA TCC AGG CCG TCA A antisense	60	300
NOD2 mouse	GCT GCC AAT CTT CAC GTC GTC sense TAA GTA CTG AGG AAG CGA GAC TGA antisense	60	180
NOD1 human	GTA AAG GTG CTA AGC GAA GA sense TCT GAT TCT GGA TAA GCC AT antisense	55	470
NOD2 human	AAC CAC TCT CTG TGC GGA CT sense CCT GTT CAG AGA AGC CCT TG antisense	60	350
p27 mouse	GAG GGC AGA TAC GAA TGG CAG sense CTG GAC ACT GCT CCG CTA ACC antisense	60	238
HPRT mouse	AAC TCT CGA AGT GTT GGA TAC AGG sense CTT GCG CTC ATC TTA GGC TTT G antisense	55	168
β-actin	ACG GGG TCA CCC ACA CTG TGC sense CTA GAA GCA TTT GCG GTG GAC GAT G antisense	60	660

Table 2: 1st column: name of the gene; 2nd column: sense and antisense primer sequences; 3rd column annealing temperature; 4th column: expected fragment size

The amplified fragments were electrophoresed in 1.5% agarose gel, in 1 x TBE buffer for 15-20 minutes at 80 V and then visualized by ethidium bromide under UV light. The 1 kb Plus DNA Ladder marker was used to determine the fragment size. In table 2, the primers used are listed together with the corresponding sequence, annealing temperature and expected length of the amplified fragment. Each sequence was checked with the NCBI BLAST program in order to exclude eventual annealing with other genes different from the ones studied. All primers were synthesized by MWG Biotech, reconstituted with sterile distilled water to a concentration of 100 µM and stored at -20 ° C.

3.5 Luciferase reporter gene assay

3.5.1 Plasmids

Plasmids are circular DNA molecules used for studying the control of gene expression and investigating the regulatory elements and cell signaling. Plasmids used in molecular biology contain an antibiotic resistance gene, which helps to select only the host bacteria resistant to a certain antibiotic during the transformation step. There are expression and reporter plasmids. An expression plasmid is used to introduce a specific gene into a target cell. Once the expression vector is inside the cell, the protein that is encoded by the gene is produced by the cellular transcription and translation machinery. The expression plasmids used in this study are: NOD2 expression plasmid pcDNA3 HA-NOD2 and control plasmid pcDNA3 HA (provided by Dr. Gabriel Nunez, University of Michigan Medical School, Ann Arbor, MI, USA). As an expression plasmid for NOD1 was not available, the experiments of this study related to overexpression were performed only with the NOD2 expression plasmid. Reporter plasmids contain a fusion gene, in which the promoter of an endogenous gene is coupled to the reporter gene, whose product is easier to detect. If the endogenous gene promoter is "turned off", neither the endogenous gene nor the reporter gene is transcribed. Likewise, if the promoter for the endogenous gene is activated, then the reporter gene is also transcribed. All the reporter plasmids in this study contain the reporter luciferase. Upon addition of its substrate luciferin, the release of light is catalyzed by the firefly luciferase enzyme. The emission peaking at 562 nm is easily measured in a luminometer and correlates with the transcription of the gene promoter coupled to luciferase in a certain plasmid. The reporter plasmids used in this work are: NF-κB-Luc (provided by Dr. Bell, Mayo Clinic, Rochester, MN, USA), POMC-Luc (Dr. M. Low, Oregon Health and Science University, Portland, USA), p53-Luc (Mercury pathway profiling system, Clontech Laboratories, Inc., Palo Alto, CA, USA), NurRE-Luc (Dr. J. Drouin, Montreal, QC, Canada) and AP-1-Luc (Stratagene, La Jolla, California, USA).

3.5.2 RNA interference

Regulation of eukaryotic gene expression occurs at different stages of protein biosynthesis: at the transcriptional, RNA processing and translational levels and at the level of protein maturation/degradation. The post-transcriptional level has recently

attracted much attention because of the discovery of the phenomenon called RNA interference (RNAi) [123]. RNAi is initiated by the enzyme Dicer (an endoribonuclease RNase III), which cleaves long double-stranded RNA (dsRNA) molecules into short fragments of 21–25 base pairs with a few unpaired overhang bases on each end. These short double-stranded fragments are called small interfering RNAs (siRNAs). The siRNAs are then separated into single strands and integrated into an active RISC (RNA induced-silencing complex) complex. After integration into RISC, the siRNAs base-pair to their target mRNA and induce cleavage of the mRNA, thereby preventing it from being used as a translation template. The whole process of interference is shown in figure 11.

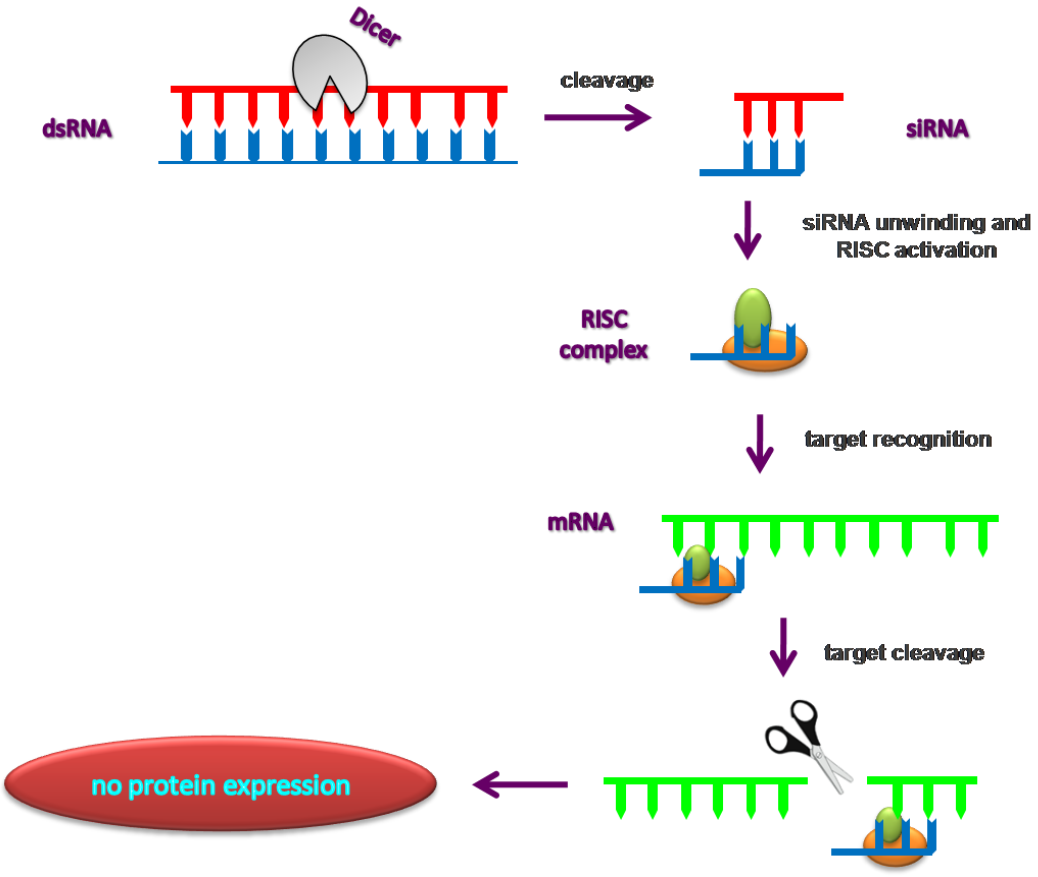


Fig. 11: RNA interference process

SiRNA for targeting endogenous NOD1 and NOD2, as well as a non-targeting siRNA, used as a control (siRNA control) sequence, were obtained from Santa Cruz Biotechnology. AtT20 cells were transfected using 13 nM siRNA for 24 hours. The transfection experiments were performed as described in the following section.

3.5.2 Transfections

Lipofection was successfully used for transfections. The principle of lipofection is based on the formation of a DNA-liposome complex between the positively charged lipofectamine and the negatively charged DNA, which subsequently leads to the fusion with a cell membrane and the uptake of the DNA into the cell.

The day before the experiment, AtT20 cells were seeded in 24-well plates at a density of 2 x 10^5 cells per well using 500 µl of normal DMEM medium containing 10% FCS with all nutrients. To prepare the transfection mix, Opti-MEM® Medium was used, as this reduced serum media is ideal for cationic lipid based transfections. For each well a mixture was prepared containing 0.8 µg DNA (in total), 2 µl of Lipofectamine 2000, and since NOD expression is restricted to the cytoplasm, the stimulus for NOD1 and NOD2 were also included into the mix in the desired concentration. The RSV-β-gal construct was also included to the mix to verify later in parallel the efficiency of the transfection (described below). The mix was left for incubation under the laminar flow for 20 minutes. Meanwhile, 100 µl of medium from each well was removed from the transfection plate. Following the incubation time of the Lipofectamine 2000 mixture, 100 µl of the mix was added to each well. According to the experiment performed, the transfection and stimulation time varied. After the transfection and stimulation time, the supernatant was removed and the cells were washed with PBS. 100 µl of lysis buffer (diluted 1:5 in water) was added to the wells and stored on the shaker at room temperature for 15 minutes. Afterwards, the plate was stored at -80° C just until the cells got frozen. After thawing, the cells were transferred to Eppendorf tubes and centrifuged for one minute. 20 µl of the supernatant were plated in a non-transparent 96-well plate, which was placed in the TriStar machine that automatically added 50 µl of luciferin to each well and measured the luciferase activity.

The transfection efficiency was determined in parallel by performing the β-galactosidase assay. 20 µl of the supernatant, 30 µl of distilled water and 50 µl of ONPG buffer were

placed on an ELISA-reader plate. The light protected plate was stored in the incubator for 10-60 minutes until the solution began to turn yellow and the absorption could be measured. Results are shown as percentage with respect to control plasmids or basals, and are ratios of luciferase to β-gal activity, in order to normalize the results according to the transfection efficiency in each well. All experiments were carried out in triplicate and repeated three times independently.

3.6 Biochemical Methods

3.6.1 Protein extraction

To perform SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western immunoblotting, AtT20 cells were seeded on 6-well plates with a density of 1 x 10^6 per well. The next day, they were starved for 24 hours and afterwards stimulated with 30 µg/ml iE-DAP and 30 µg/ml MDP for the desired time. After the stimulation time elapsed, the supernatant was removed from the plate, the cells were washed with PBS, scraped with 500 µl PBS and transferred to Eppendorf tubes. Following, they were centrifuged at 1400 rpm for 10 minutes at 4 °C. The supernatant was discarded and the protein extracted through breaking the cell membranes by adding 150 µl of RIPA lysis buffer, 1:10 PhosST*O*P Phosphatase Inhibitor Cocktail and 1:100 protease inhibitor cocktail, all diluted in distilled water. The whole procedure was performed on ice. The samples were sonicated before they were used for SDS-PAGE or stored at -20°C.

3.6.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

After the final sonication step of the protein extraction the samples were centrifuged, after which the supernatant contained the proteins. 15 µl of proteins were mixed with 5 µl of Roti®-Load1 loading buffer and boiled at 95°C for 5 minutes. Electrophoresis was carried out using a pre-cast Tris-Glycine 10% gel in an Invitrogen XCell SureLock™ Mini-Cell electrophoresis apparatus at a constant voltage of 130V for 1-1.5 hours. This separates the proteins by size and in order to monitor progress of protein electrophoresis and molecular weight of blotted proteins, the Marker Precision Plus Protein™Standards was used.

3.6.3 Western immunoblotting

The Western Blot is a technique for detection of proteins in different samples such as tissues or cell extracts, serum, liquor or cell culture supernatants. After separation of the proteins by electrophoresis, they are transferred to different types of membranes and detected by various methods, among them by immunological methods. The latter allows the measurement of the relative amount of a specific protein present in the samples. This is possible by using a primary specific antibody directed against the studied protein, which is detected by a secondary antibody (horseradish peroxidase-conjugated) and visualized on a nitrocellulose membrane, after incubation with a substrate developing a luminescent product. In this study PVDF membranes were used because of their higher binding capacity compared to nitrocellulose membranes. Before usage, the PVDF (Polyvinylidene fluoride) membranes were first pretreated by soaking them for 15 seconds in methanol, then 2 minutes in distilled water and at least 5 minutes in transfer buffer. The "semi-dry"- method, by using the Invitrogen Novex® Semi-Dry Blotter, is a system that allows efficient and rapid electrophoretic transfer of proteins. The blotting is performed with plate electrodes in a horizontal configuration, sandwiching gel and membrane between sheets of transfer buffer soaked filter paper that functions as the ion reservoir. During electrophoretic transfer at 150 mA for one hour, negatively charged molecules migrate out of the gel, move towards the positive electrode, and encounter the membrane, where they bind. Afterwards, the membrane was soaked again for 15 seconds in methanol, 2 minutes in distilled water and stored on a filter paper to dry. Next, the Ponceau S solution dye was used to make a rapid reversible stain of the membrane to verify that the amount of protein was equal in every sample. The staining was easily removed by water washing, followed by a blocking step for one hour at room temperature in 5% milk powder, diluted in 1x TBST. Afterwards, the primary antibody was prepared in the desired dilution in 5% BSA and incubated overnight at 4°C. After three washes with TBST the following morning, the membrane was incubated with the secondary antibody diluted in 5% milk powder for one hour at room temperature. The secondary antibody is conjugated to Horseradish Peroxidase, an enzyme which allows visualization of the reaction. The detection was carried out by using ECL Western Blotting Detection System. The acridan-based substrate used in this system releases a high level sustained output of light, which can be detected by an x-ray film exposed to the membrane in an autoradiography cassette, to detect the light given off by the

enzyme reaction. The film was removed and developed to visualize the immunoreactivity bands. The bands were present wherever there was a protein-primary antibody-secondary antibody-enzyme complex, or in other words, wherever the protein under investigation was. In order to confirm the equal protein amount in every sample, the procedure was repeated using antibodies detecting the total protein levels, or the anti-β-actin antibody, an antibody against a highly conserved protein found in all eukaryotic cells.

3.6.4 Hormone measurement by RIA (Radioimmunoassay)

RIA (Radioimmunoassay) is a highly sensitive and quantitative technique used for the measurement of substances such as enzymes, proteins and hormones that exist in very low concentrations. In this study, the RIA was used to measure the concentration of rat and mouse ACTH levels secreted in the medium by primary rat pituitary cultures and AtT20 cells. RIA uses radiolabeled antigens (Ag) to detect Ag:Ab (antibody) reactions. The procedure follows the basic principle of radioimmunoassay, where there is competition between a radioactive and a non-radioactive antigen for a fixed number of specific antibody binding sites. The antigens are labeled with the I^{125} (iodine-125) isotope, and the presence of Ag:Ab reactions is detected using a gamma counter.

The first step for starting the RIA is developing an antibody that is highly specific for the hormone being measured. An N-terminal specific antibody against rat ACTH was raised in rabbits using an antigen produced by the two-step carbodiimid method [124]. Standards were purchased from Bachem. A small quantity of the antibody was mixed with a certain quantity of the sample (cell culture supernatant) containing the hormone to be measured. At the same time, a certain amount of tracer (standard antigen labeled with the radioactive isotope I^{125}) was added to the mixture. The samples were incubated one hour at 37°C, allowing time for the hormone (Ag) to bind to the antibody. The mixture was prepared in such quantities that there were not enough antibodies to bind with both the labeled hormone and with the hormone to be measured, so the natural hormone and the labeled hormone competed for binding sites. The quantity of each hormone bound was proportional to their concentration, and the amount of labeled hormone (tracer) bound to the specific antibody was inversely proportional to the concentration of the natural hormone. After binding had reached the equilibrium, the

quantity of radioactive hormone bound to the antibody was measured in a gamma counter. As explained above, the amount of radioactivity present in the test was inversely proportional to the amount of hormone in the sample. Quantification of the unknown free hormone in the sample was achieved by comparing their activity with a standard curve prepared by using increasing amounts of known concentrations of the hormone. To study the effect of NOD receptor ligands on ACTH production, the primary rat pituitary cell culture and the AtT20 cells were serum deprived overnight, stimulated for 24 and 48 hours with 30 μg/ml of iE-DAP and 30 μg/ml of MDP in medium containing 2% FCS, and afterwards the supernatants were collected for ACTH analysis by RIA.

3.6.5 Single Immunohistochemistry (IHC)

The technique of IHC is useful to visualize the localization and expression intensity of a specific protein within a cell or tissue. The basic principle of this method (Fig. 12) is the ability of antibodies to specifical recognize particular subsequences of a protein, called epitopes. The technique involves three steps. In the first step an unlabeled primary antibody, specific for the protein of interest, binds to its target in fixed cells or tissue slices. In the second step a biotinylated secondary antibody, raised against the antibodies of the animal in which the primary antibody was produced, binds to the primary antibody. In the third step a complex of avidin-biotin peroxidase is created. Biotin, a low molecular weight vitamin, can be conjugated to a variety of biological molecules such as antibodies. Avidin, a large glycoprotein, is labeled with peroxidase enzyme and has a very high affinity for biotin. The peroxidase produces a brown precipitate actin on the chromogen diaminobenzidine (DAB) and therefore makes the reaction visible at microscope.

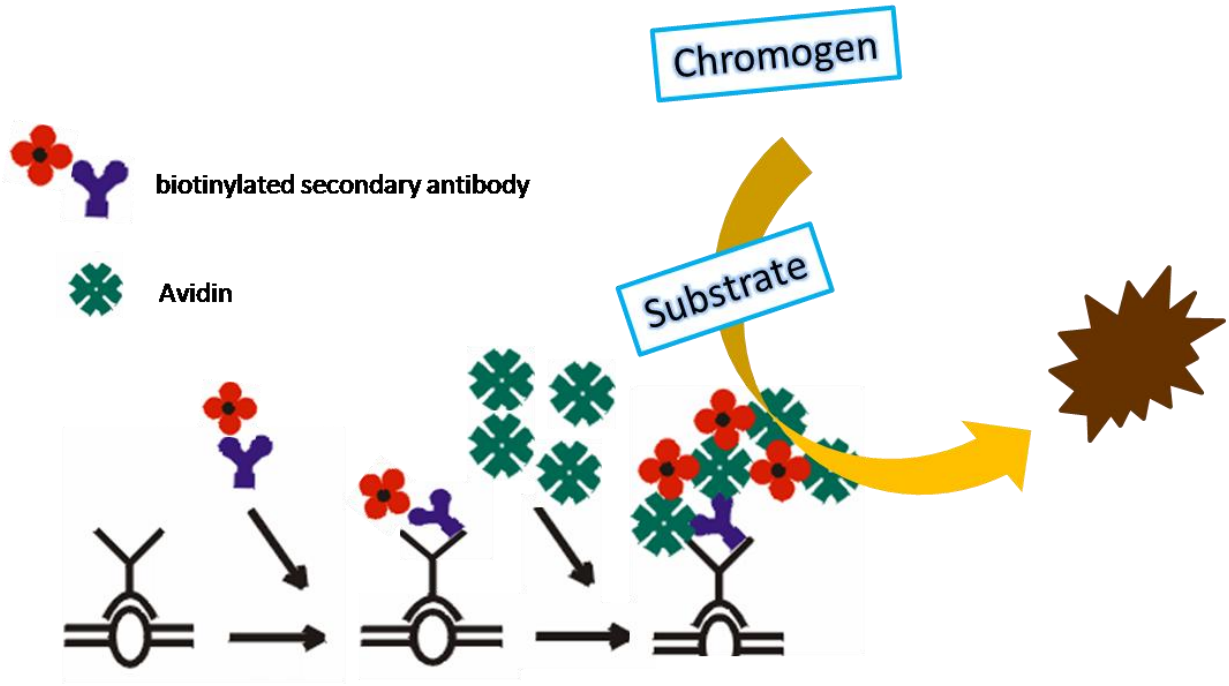


Fig. 12: The principle of IHC. The antigen of interest is recognized by a specific primary antibody, which is in turn recognized by a biotinylated secondary antibody. The biotin is bound by avidin, conjugated with peroxidase, which is using respectively DAB as substrate and catalyzes a chromogenic reaction yielding a brown coloured product.

AtT20 cells were seeded on slides and cultured for two days before they were fixed in 4% paraformaldehyde (PFA), freshly prepared in PBS, and stored in 96% ethanol at 4° C until use. After a wash in 1 x TBS for 5 minutes, slides were first blocked for 30 minutes in serum (diluted 1:10 in 1 x TBS buffer pH 7.6) of the same animal in which the specific biotinylated secondary antibody was raised. Afterwards, the primary antibody was applied and the slides were left overnight at 4°C. The next day, after three washes in TBS buffer, the biotinylated secondary antibody was added at room temperature for 30 minutes. After three further washes in TBS buffer, the slides were incubated with the avidin-biotin-peroxidase complex (ABC-complex) at room temperature. The ABC complex was prepared in Tris buffer at least 30 minutes in advance to allow the complex formation. The development was performed using freshly prepared 1 mg/ml DAB with 0.01% hydrogen peroxide. DAB incubation time was different for each antibody and was decided by checking the brownish precipitate. Since DAB is light-sensitive, these final steps were performed in darkness. After three more washes, the slides were finally counterstained for 15 minutes in toluidine blue, which stains nuclei in light blue. Excess

of color was removed with two washes in distilled water and one final wash in 70% ethanol supplemented with acetic acid. Next, two dehydration steps in 96% ethanol and 100% ethanol was followed before the slides were fixed in Roti-Histol and coverslipped using Roti-Histokitt.

3.7 Statistics

Hormone secretion assays were performed in quadruplicate wells, cell proliferation and transfection experiments were all performed in triplicate wells and results are expressed as mean ± standard error. For statistical analyses of stimulation experiments, the mean values were compared by one-way ANOVA. P values smaller than 0.05 were considered significant and marked with asterisks. For Western Blot bands and RT-PCR bands analysis, the software ImageJ 1.42 was used. After calibration, the software is able to convert the intensity of bands into numeric values, which then could be shown as a bar diagram.

4. Results

4.1 Expression of NOD1 and NOD2 in the pituitary gland

4.1.1 NOD proteins in the pituitary tumoral cell line AtT20

In my diploma thesis, I previously examined the expression of NOD1 and NOD2 in the pituitary tumor cell line AtT20. I found the expression of these two intracellular receptors by RT-PCR and immunohistochemistry.

4.1.2 NOD expression in normal human pituitaries and human pituitary adenomas

Recently, NOD1 and NOD2 were shown to be implicated in tumorigenesis in various types of cancer [76, 78]. Since it was already demonstrated that immune receptor activation modulates pituitary tumor progression [79], the expression of NOD1 and NOD2 was also studied in the pituitary gland and in human pituitary adenomas. In four normal pituitary samples the expression of these two receptors could be detected by RT-PCR (Fig. 13 a) and IHC (Fig. 13 b).

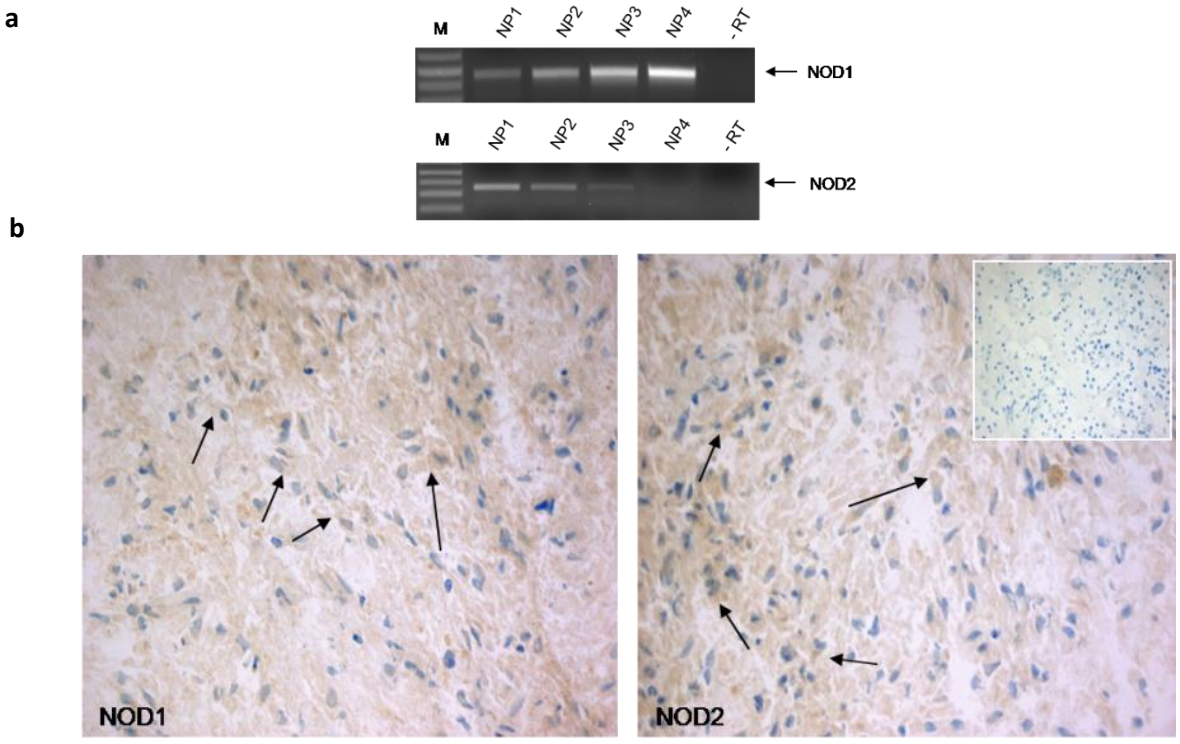


Fig. 13 a + b: Detection of NOD1 and NOD2 in the human pituitary gland.

a) mRNA expression levels of NOD molecules in human pituitaries (NP: normal pituitary).

b) Arrows indicates NOD1 and NOD2 positive cells (brownish) in human pituitary

Next the importance of the expression in pituitary adenomas was examined and assessed. A detailed description of the human pituitary adenomas used is given in table 1 (page 36). Interestingly, about half of the studied human pituitary adenomas had higher levels of expression when compared to the normal pituitary gland (Fig. 14). Somatotropinomas and corticotropinomas showed the highest and non functioning pituitary adenomas the lowest mRNA levels. The mRNA levels obtained were compared with the expression of the β-actin housekeeping gene. The normalized results could finally be presented as a bar chart.

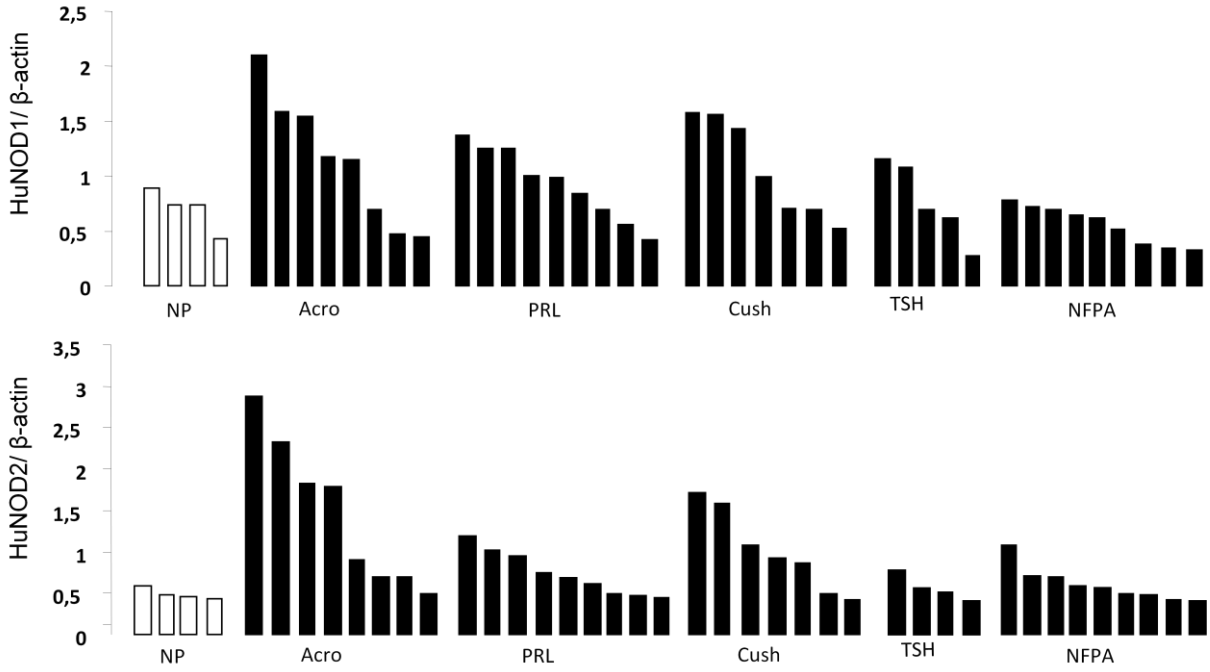


Fig. 14: Bar chart of human NOD1 and NOD2 mRNA expression values. The optical density was calculated by ImageJ based on the intensity of the RT-PCR bands. Abbrevations: NP (normal pituitary), Acro (somatotropinomas), PRL (prolactinomas), Cush (corticotropinomas), TSH (thyreotropinomas), NFPA (non functioning pituitary adenomas)

All together the results indicate that NOD immune receptors are expressed in tumoral pituitary AtT20 cells as well as in normal pituitaries and human pituitary adenomas. This outcome suggests that these receptors are functional in the pituitary gland, and the elevated mRNA expression levels in a proportion of pituitary adenomas indicate a possible importance of these receptors in tumorigenesis.

4.2 NOD receptor activation and its impact on POMC activity and ACTH secretion

As dysregulation of normal feedback processes seems to occur in adrenocorticotropic (ACTH)-secreting tumors, the physiological relevance of NOD expression in terms of hormone production was evaluated. In addition to ACTH secreting AtT20 cells, cells of normal rat pituitaries were also included in this set of experiments.

4.2.1 NOD ligands downregulate POMC activation and ACTH secretion in AtT20 cells

POMC (Proopiomelanocortin) is synthesized by corticotrope cells of the anterior pituitary gland. It undergoes post-translational processing, which is essential for the production of bioactive peptides like ACTH. As shown by transfection of AtT20 cells with the POMC-Luc plasmid, treatment with 30 µg/ml of iE-DAP and MDP inhibited POMC promoter activity (Fig. 15).

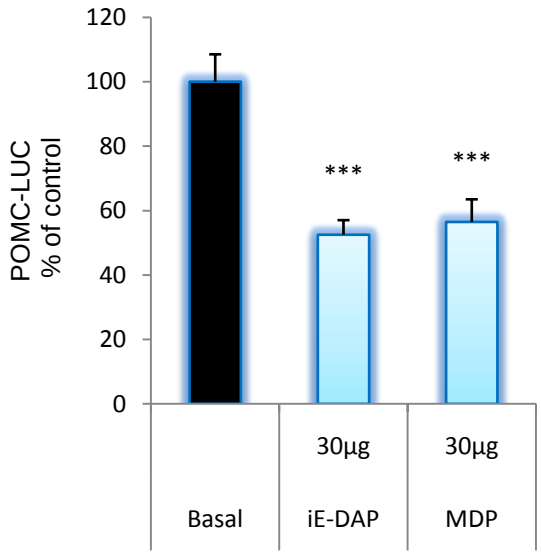


Fig. 15: NOD ligands inhibit POMC luciferase activity in AtT20 cells. AtT20 cells were transfected with POMC-LUC plasmid and stimulated with 30 µg/ml of iE-DAP and MDP. After 24 hours, luciferase activity was measured. Values are presented in percentage of control and as the mean ± SE (*** $P < 0.001$).

By overexpressing NOD2 the inhibitory effect of NOD receptor activation on the POMC promotor could be repeated (Fig. 16).

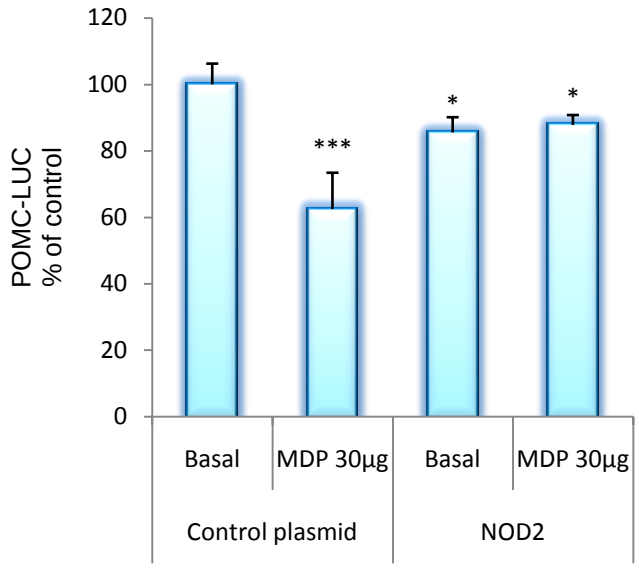


Fig. 16: Decreased POMC luciferase activity in NOD2 overexpressed AtT20 cells. AtT20 cells were transfected with control plasmid or NOD2 expression vector along with POMC-LUC with or without 30 µg/ml of MDP. 24 hours post-transfection, luciferase activity was measured. Values are presented in percentage of control and as the mean ± SE (* $P < 0.05$ and *** $P < 0.001$).

POMC transcription depends on expression of Nur elements, which upon activation bind to the target DNA element on the POMC promoter [125]. The addition of NOD2 overexpression plasmid resulted in a strong inhibition of NurRE luciferase activity (Fig. 17).

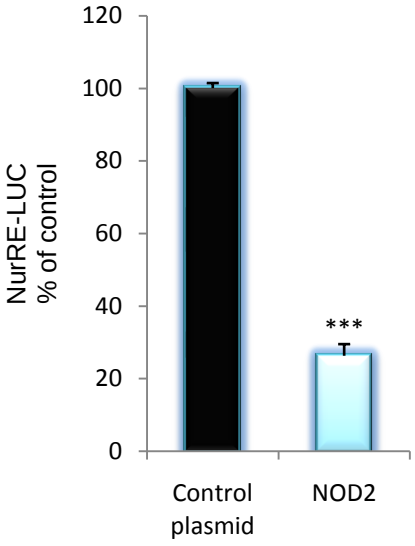


Fig. 17: Overexpression of NOD2 inhibited NurRE luciferase activity in AtT20 cells. AtT20 cells were transfected with control plasmids or NOD2 expression vector along with NurRE-Luc plasmid. 24 hours post-transfection, luciferase activity was measured. Values are presented in percentage of control and as the mean ± SE (*** $P < 0.001$).

After stimulation of AtT20 cells with 10 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml of iE-DAP and MDP for 48 hours and removal of its supernatant, the RIA (Radioimmunoassay) technique was used to determine the concentration of hormones, in this particular case the concentration of ACTH. The ligands of NOD1 (iE-DAP) and NOD2 (MDP) clearly decreased the ACTH levels when compared to basal levels of the unstimulated samples (Fig. 18).

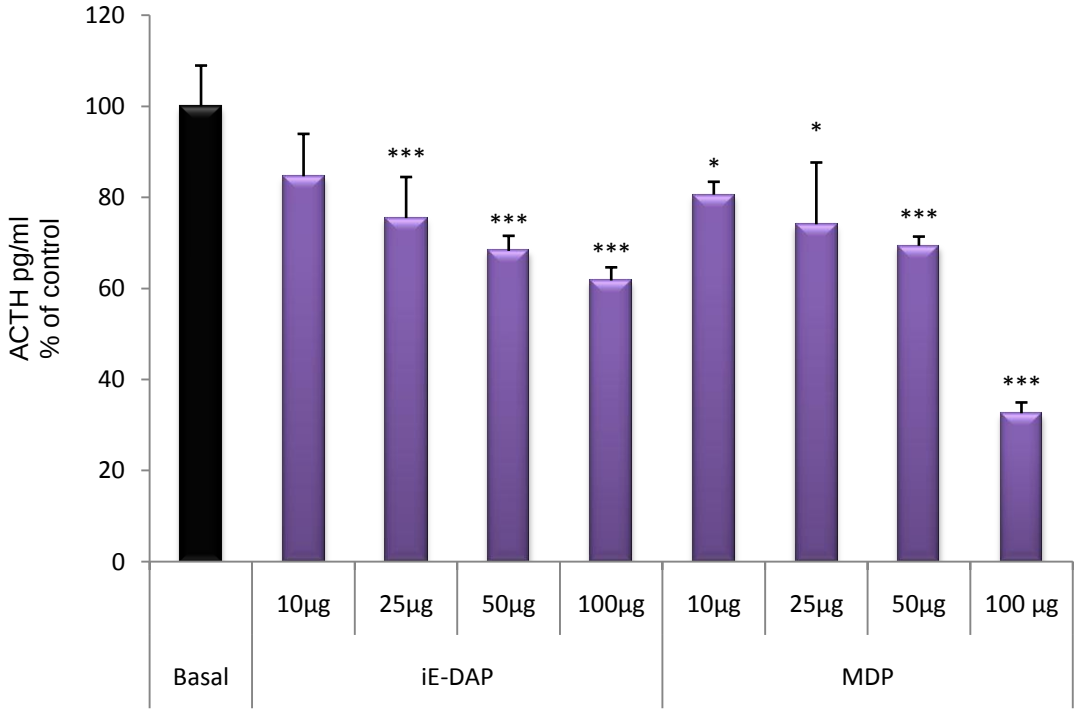


Fig. 18: ACTH levels in with NOD ligands treated AtT20 cells assessed by Radioimmunoassay. AtT20 cells were left stimulated or unstimulated for 48 hours, followed by the RIA measurement. Values are presented in percentage of control and as the mean ± SE (* $P < 0.05$ and *** $P < 0.001$).

4.2.2 ACTH secretion of normal rat pituitaries is not influenced by NOD receptors

Rat pituitary cells were handled as described in chapter 3.3.1 and stimulated for 24 and 48 hours in a similar manner as AtT20 cells to examine the effect of NOD receptor activation on ACTH secretion. After the stimulation time elapsed, the supernatant was removed and evaluated by RIA. CRH and Forskolin are well-known activators of ACTH secretion in corticotropic cells. Therefore, as a control of potent cells, 100 nM of CRH and

5 µM of Forskolin were also included in the experiment. The results demonstrate that NOD receptors have no impact on ACTH secretion in normal rat pituitaries (Fig. 19 a + b).

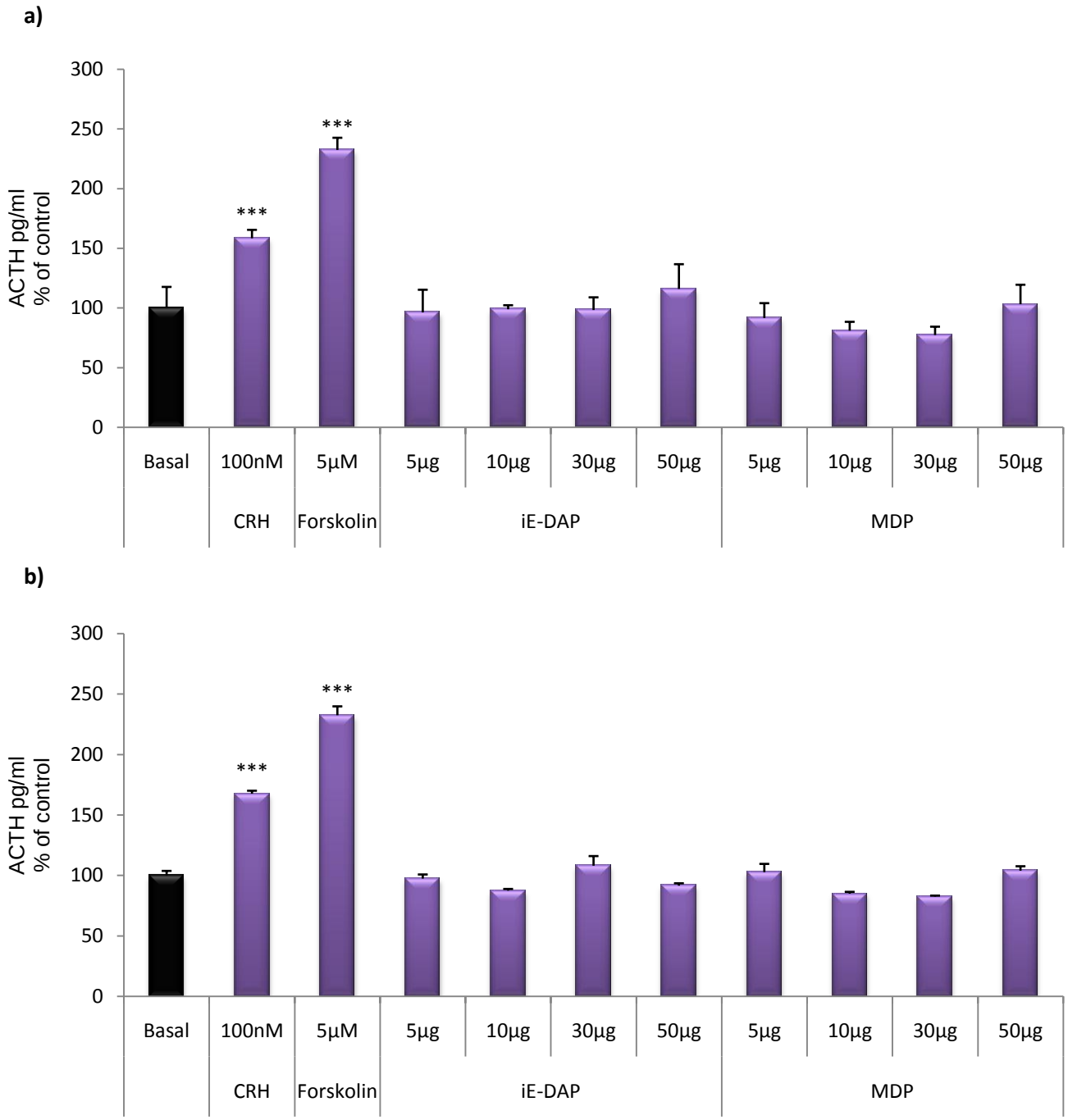


Fig. 19 a+b: ACTH secretion in normal rat pituitaries. a) ACTH levels assessed by RIA after 24 hours of stimulation with 100 nM CRH, 5 µM Forskolin, 5 µg/ml, 10 µg/ml, 30 µg/ml and 50 µg/ml of iE-DAP and MDP; b) ACTH levels assessed by RIA after 48 hours of stimulation with 100 nM CRH, 5 µM Forskolin, 5 µg/ml, 10 µg/ml, 30 µg/ml and 50 µg/ml of iE-DAP and MDP. Values are presented in percentage of control and as the mean ± SE (*** $P < 0.001$).

To sum up, it could be said that on the one hand NOD receptor activation suppressed POMC promoter activity and subsequently the ACTH secretion in AtT20 cells, but on the other hand no changes could be observed in ACTH secretion in normal rat pituitaries after treatment with the specific ligands of NOD1 and NOD2.

4.3 NOD receptors are involved in cell proliferation

Whereas in immune cells NOD1 and NOD2 act as sensors of bacterial invasion [126], their role in tumor cells is largely unknown. Since it appears that NOD signaling is active in the ACTH secreting cell line AtT20 and in pituitary adenomas, the effect of these intracellular receptors on proliferation was further investigated.

4.3.1 NOD ligands increase proliferation in AtT20 cells

Both NOD1 ligand iE-DAP and NOD2 ligand MDP stimulated the proliferation of AtT20 cells. The cells were treated with 10 µg/ml, 30 µg/ml and 50 µg/ml of each stimulus for 48 hours, and the effect was carried out by the WST-1 proliferation assay. Both ligands significantly increased the proliferation in AtT20 cells, shown in figure 20.

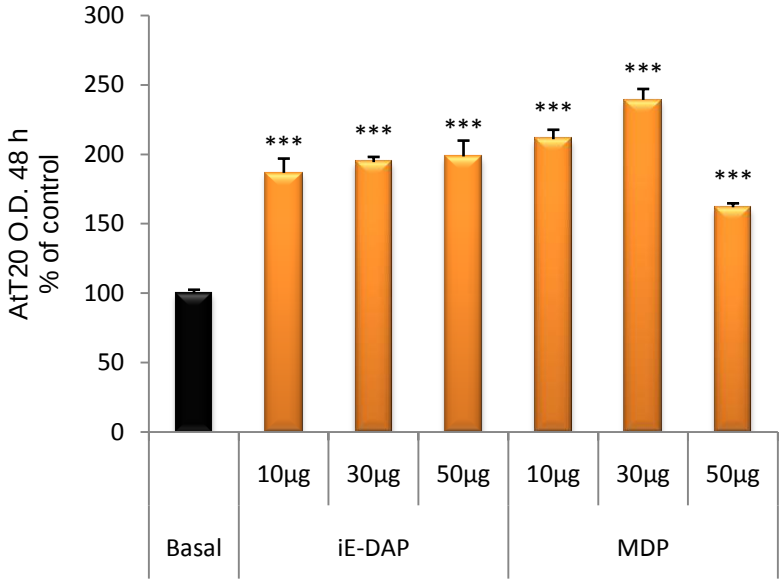


Fig. 20: AtT20 WST-1 proliferation assay. Cell proliferation was examined in untreated cells and treated cells with 10 µg/ml, 30 µg/ml and 50 µg/ml of both, iE-DAP and MDP. Proliferation was measured 48 hours after treatment by WST-1 assay. Values are presented in percentage of control and as the mean ± SE (*** $P < 0.001$).

As already described in the introduction, the PI3K pathway plays crucial roles in many cellular processes, including proliferation. Proliferation can be suppressed by the PI3K inhibitors Wortmannin and LY294002 [127]. Thus, it would be interesting to elucidate the role of PI3K in AtT20 cells` proliferation. As the proliferation is not dose-dependent but the 30 µg/ml concentration of each stimulus seemed to be the most suitable one, this concentration was chosen for further experiments. In the first set of the experiment the cells were treated with both ligands. Additionally, some of them were left untreated to show the basal condition. The second set was treated the same, but additionally with 5 µM of the PI3K inhibitor LY294002. As represented in figure 21, the proliferative effect induced by the ligands was inhibited.

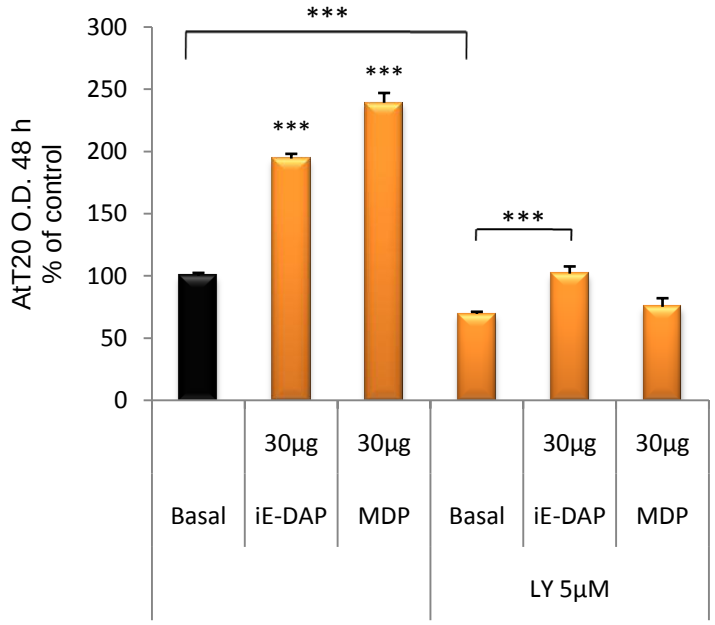


Fig. 21: Effect of PI3K inhibitor LY294002 on AtT20 cells. Cells were pre-treated with serum-free medium and 5 µM of LY294002 for one hour and then stimulated with iE-DAP (30 µg/ml) and MDP (30 µg/ml) for 48 hours. Proliferation was measured by WST-1 assay. Values are presented in percentage of control and as the mean ± SE (*** $P < 0.001$).

In order to complete and circumstantiate the result obtained with the LY294002 inhibitor, a second PI3K inhibitor was included in the experiments. The cells were treated with 1 nM Wortmannin to explore and confirm the effect on AtT20 cells` proliferation. As expected, Wortmannin also blocked NOD ligands induced proliferation in AtT20 cells (Fig. 22).

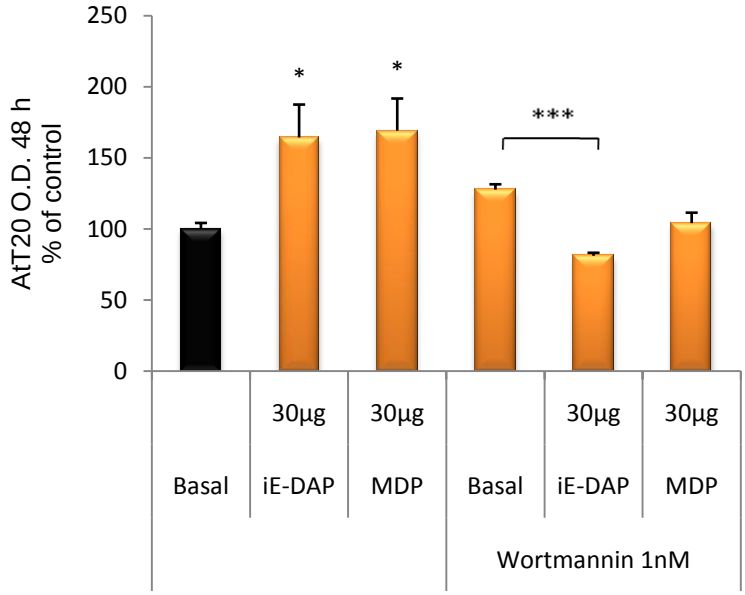


Fig. 22: Effect of PI3K inhibitor Wortmannin on AtT20 cells. Cells were pre-treated with serum-free medium and 1 nM of Wortmannin for one hour and then stimulated with iE-DAP (30 µg/ml) and MDP (30 µg/ml) for 48 hours. Proliferation was measured by WST-1 assay. Values are presented in percentage of control and as the mean ± SE (* $P < 0.05$ and *** $P < 0.001$).

As NF-κB (nuclear factor-κB) is another important pathway related to proliferation, the inhibitor Caffeic acid phenethyl ester (CAPE) was also used. CAPE is an anti-inflammatory component of propolis (honeybee resin) and reported to be a specific inhibitor of NF-κB [128]. As depicted in Fig. 23, 25 µg/ml of CAPE suppressed the proliferation induced by NOD ligands in AtT20 cells.

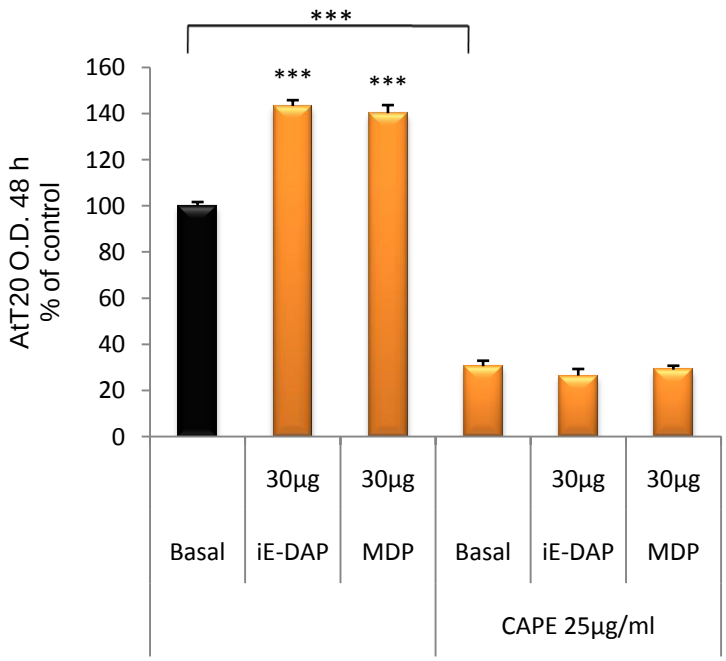


Fig. 23: Effect of NF-κB inhibitor CAPE on AtT20 cells. Cells were pre-treated with serum-free medium and 5 µg/ml of CAPE for one hour and then stimulated with iE-DAP (30 µg/ml) and MDP (30 µg/ml) for 48 hours. Proliferation was measured by WST-1 assay. Values are presented in percentage of control and as the mean ± SE (*** $P < 0.001$).

4.3.2 NOD ligands increase proliferation in pituitary adenomas

Prompted by the increased NOD mRNA levels in a subset of pituitary adenomas and the induced proliferation in AtT20 cells, the effect of NOD activation on the proliferation of pituitary adenomas was examined. Prolactinoma, non functioning pituitary adenoma, somatotropinoma and corticotropinoma cells were prepared according to chapter 3.3.1 and 48 hours after preparation, the stimulation experiment was started. The experiment was performed with the same parameters as for AtT20 cells. Proliferation triggered by the NOD ligands significantly increased in all pituitary adenomas, but generally slightly lower than in AtT20 cells. (Fig. 24 a-d).

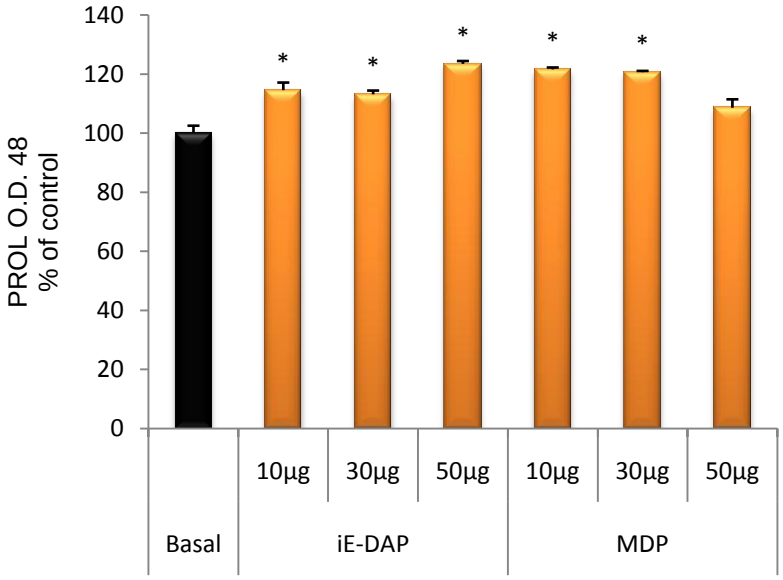


Fig. 24 a: Prolactinoma WST-1 proliferation assay. Cell proliferation was examined in untreated cells and treated cells with 10 µg/ml, 30 µg/ml and 50 µg/ml of both, iE-DAP and MDP. Proliferation was measured 48 hours after treatment by WST-1 assay. Values are presented in percentage of control and as the mean ± SE (* $P < 0.05$).

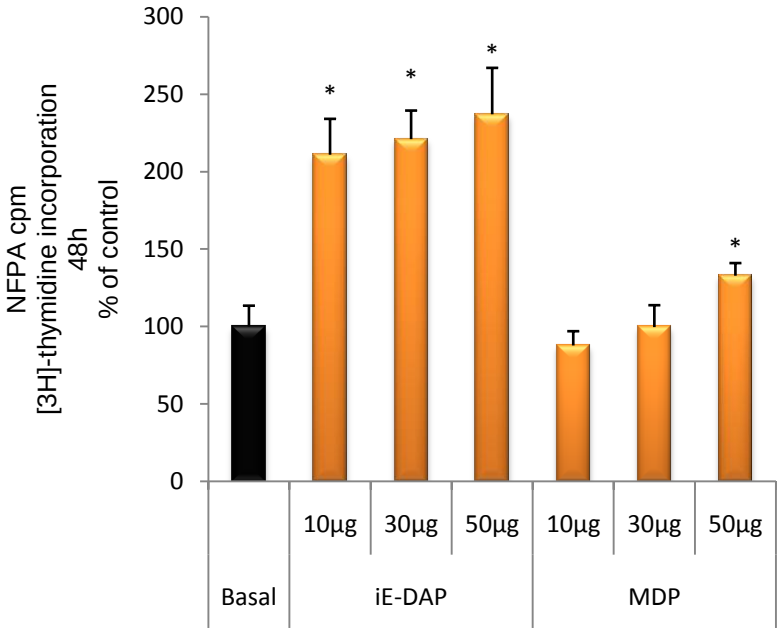


Fig. 24 b: Non functioning pituitary adenoma [3H]-thymidine incorporation assay. Cell proliferation was examined in untreated cells and treated cells with 10 µg/ml, 30 µg/ml and 50 µg/ml of both, iE-DAP and MDP. Proliferation was measured 48 hours after treatment by WST-1 assay. Values are presented in percentage of control and as the mean ± SE (* $P < 0.05$).

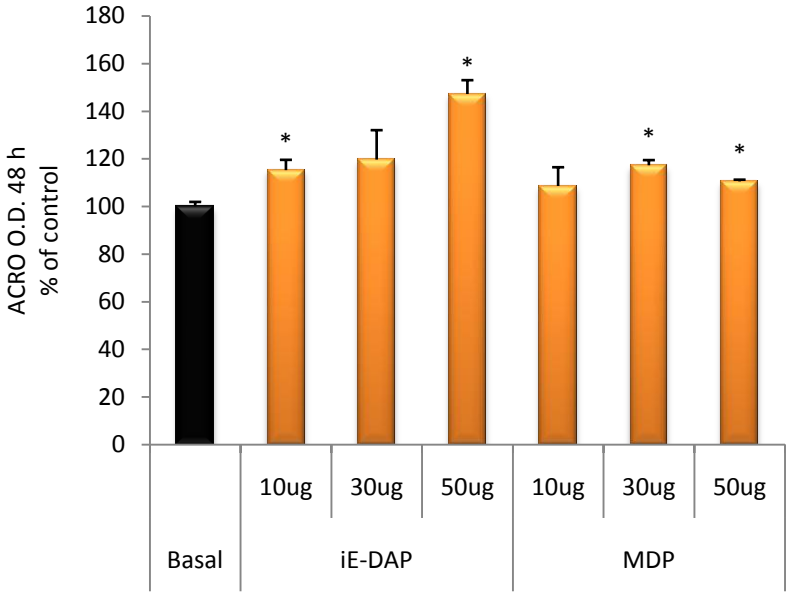


Fig. 24 c: Somatotropinoma WST-1 proliferation assay. Cell proliferation was examined in untreated cells and treated cells with 10 µg/ml, 30 µg/ml and 50 µg/ml of both, iE-DAP and MDP. Proliferation was measured 48 hours after treatment by WST-1 assay. Values are presented in percentage of control and as the mean ± SE (* $P < 0.05$).

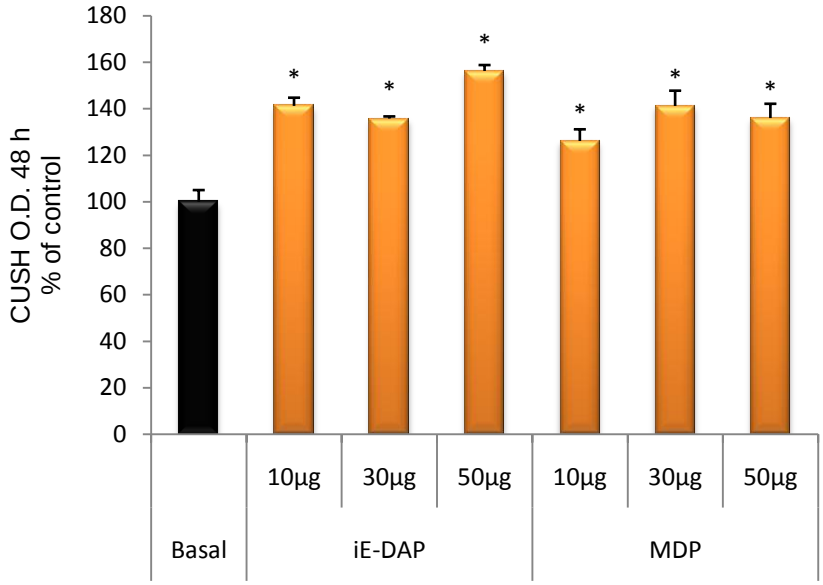


Fig. 24 d: Corticotropinoma WST-1 proliferation assay. Cell proliferation was examined in untreated cells and treated cells with 10 µg/ml, 30 µg/ml and 50 µg/ml of both, iE-DAP and MDP. Proliferation was measured 48 hours after treatment by WST-1 assay. Values are presented in percentage of control and as the mean ± SE (* $P < 0.05$).

In the non functioning pituitary adenomas only the NOD1 ligand iE-DAP in a concentration of 10 µg/ml and 30 µg/ml seemed to be not functional. Unfortunately, due to lack of material, the proliferative effect of NOD ligands on tyreotropinomas could not be investigated. The most important effect to this study is reflected by the Cushing`s associated pituitary adenomas, as this work is focused on the corticotroph cell line AtT20, which is used as a model in this study and also widely used to investigate ACTH biosynthesis and secretion.

These results suggest that NOD receptor activation stimulates proliferation and survival in pituitary cells and in pituitary adenomas. The PI3K/AKT and NF-κB pathway may act as downstream mediators on the proliferation promoting effects of NOD signaling pathway in AtT20 cells. Thus, the NOD receptor signaling pathway may specifically affect the viability of pituitary tumor cells, and the ligands of NOD may stimulate pituitary tumor cell expansion.

4.4 Cell cycle regulation by NOD receptors

Cell cycle progression of mammalian cells is controlled by a series of cyclin-dependent kinases, CDKs, and their activating cyclin subunits [50]. This process is often disturbed in tumors including pituitary adenomas [129-133]. Based on the findings that treatment with NOD ligands result in increased proliferation, the next step was to test whether NOD signaling participates in the regulation of cell cycle in AtT20 cells. The G1 to S-phase transition is often disrupted in cancers due to its intimate function in the integration of growth factor signals with the cell cycle network. Keeping this in mind, I have started to perform experiments to analyze the activity of individual proliferative signaling molecules through cell cycle. The protein extraction was carried out 24 hours after stimulation. Of the early G1 regulatory molecules, a slight increase of CDK4 protein expression after treatment with NOD ligands or overexpression of NOD2 could be observed (Fig. 25 a). As the kinase is completely inactive without its cyclin partner, the protein levels of Cyclin D1 and Cyclin D3, as corresponding CDK4 partners, were explored. Consequentially, their protein expression was also upregulated (Fig. 25 b).

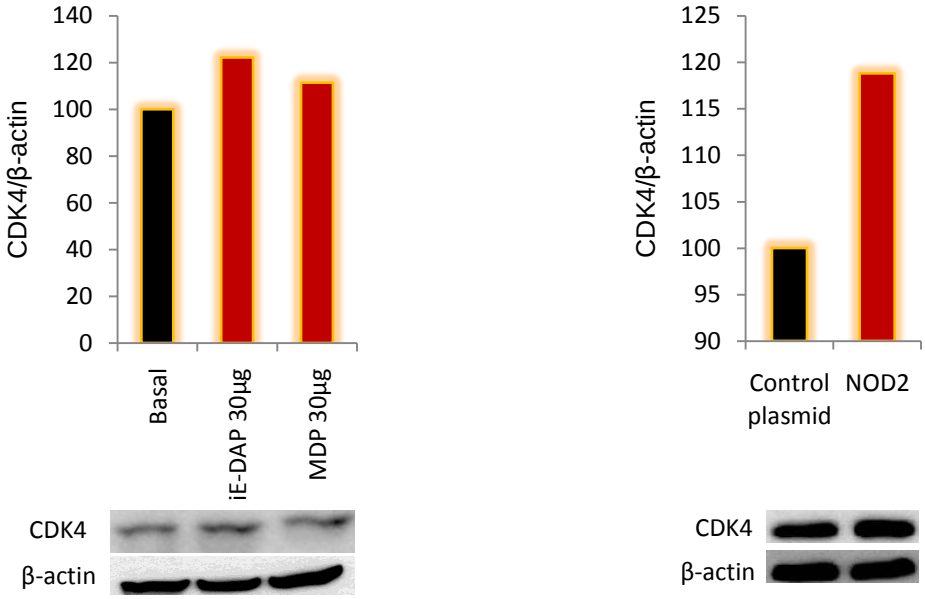


Fig. 25 a: Protein expression of CDK4 in AtT20 cells. Depicted are the increased protein levels of CDK4 after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours, and after overexpression of NOD2. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

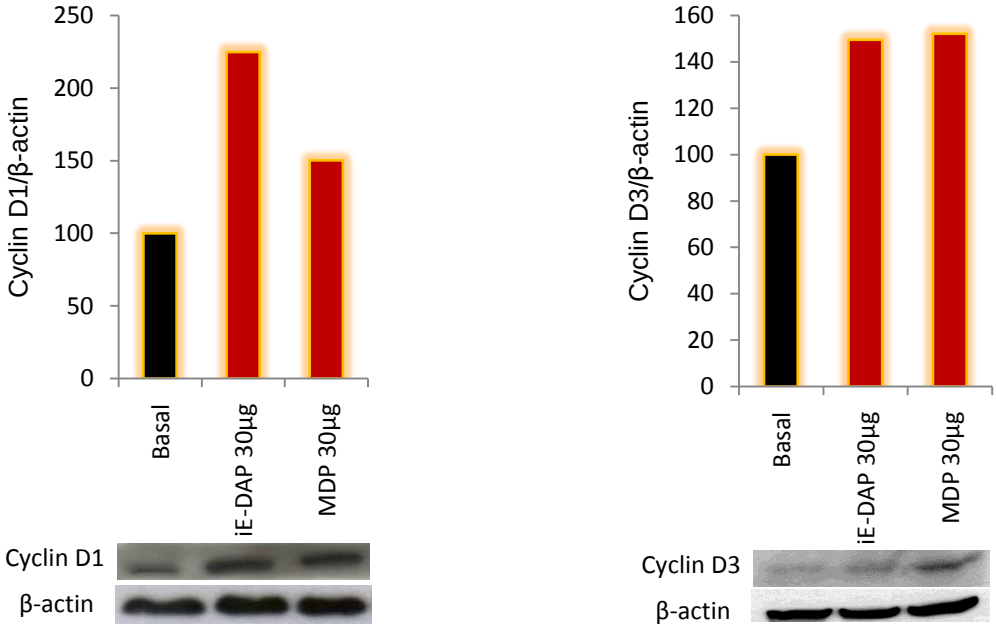


Fig. 25 b: Protein expression of Cyclin D1 and Cyclin D3 in AtT20 cells. Depicted are the increased protein levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

Additionally, the expression of CDK2 and Cyclin E, which are involved in the late G1 to S-phase progression, were also investigated. Unlike the CDK4 protein levels, the CDK2 protein expression was considerably increased after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours as shown in figure 26 a.

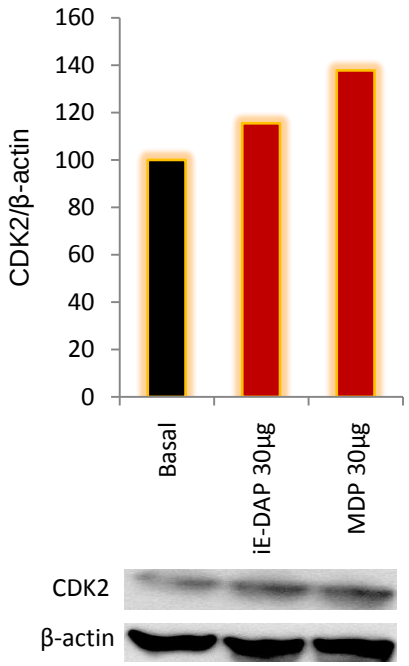


Fig. 26 a: Protein expression of CDK2 in AtT20 cells. Depicted are the increased protein levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

The protein expression of Cyclin E, which is known as the regulatory partner of CDK2, was also markedly elevated after treatment with NOD ligands on exactly the same conditions (Fig. 26 b).

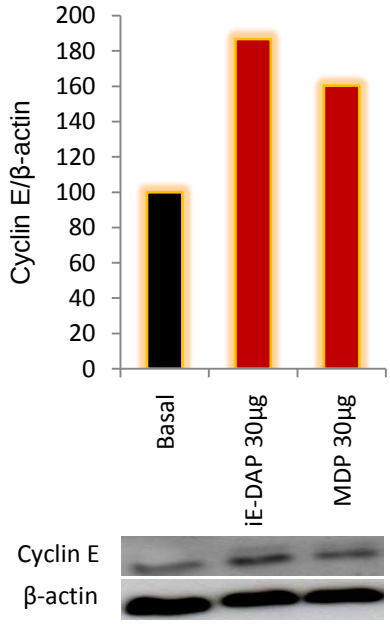


Fig. 26 b: Protein expression of Cyclin E in AtT20 cells. Depicted are the increased protein levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

As described in the introduction, the Rb (retinoblastoma) is a major component of the cell cycle. Therefore the protein level of phosphorylated Rb was explored. AtT20 cells were stimulated with 30 µg/ml of iE-DAP and MDP for 24 hours and the activation status of Rb was assessed. Simultaneously to the phosphorylation state at Ser780, the phosphorylation levels at the Ser795 residue were also examined. In figure 27 it is shown, that NOD ligands considerably increased the phosphorylation levels of this important cell cycle component at both residues, so that the cells could proceed through the cell cycle.

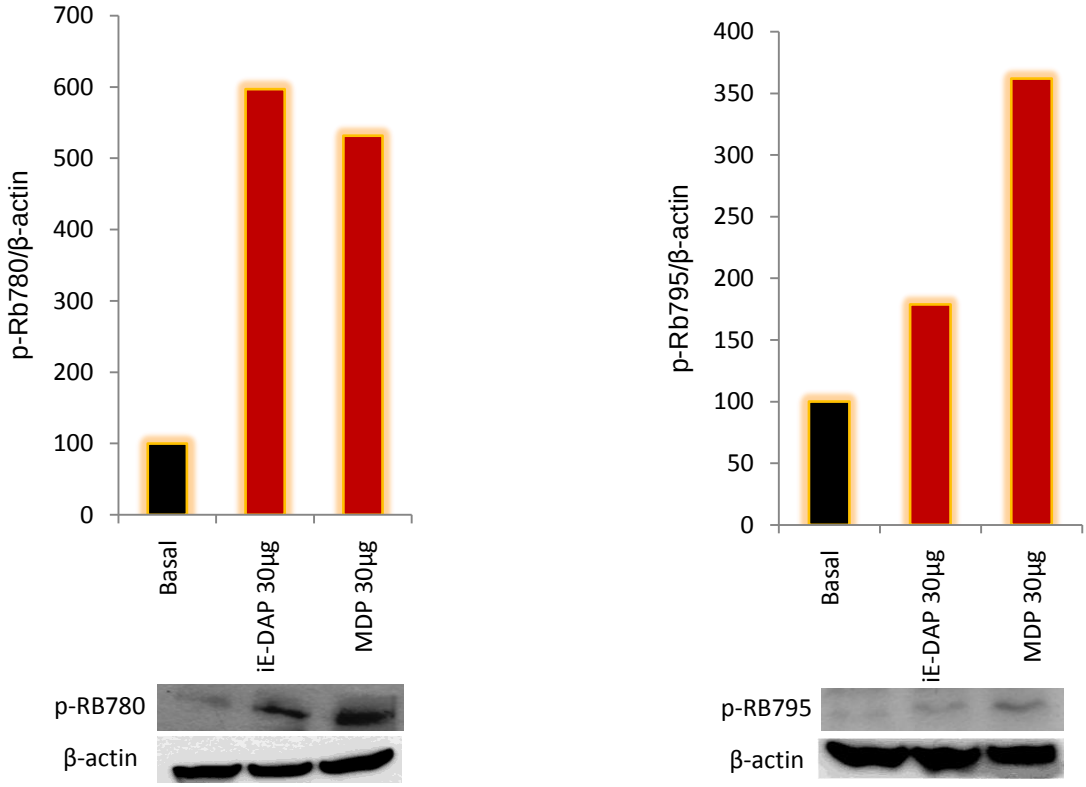


Fig. 27: Rb activation in AtT20 cells. Shown are the increased phosphorylation levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

Among the Rb, there are other important regulators of cell cycle like GSK-3β (glycogen synthase kinase-3β) and the members of the FOXO family. Thus, it was important to consider their role in this context. Both NOD ligands increased the phosphorylation of GSK-3β, even though the phosphorylation induced by iE-DAP was weak (Fig. 28).

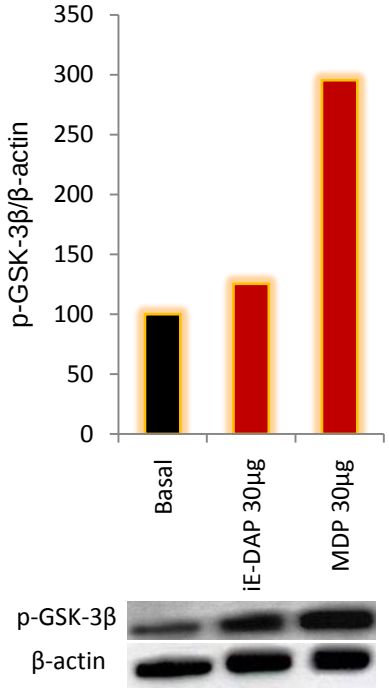
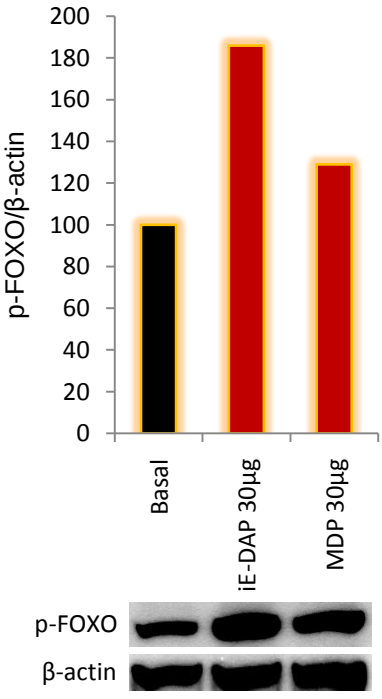


Fig. 28: GSK-3β activation in AtT20 cells. Presented are the increased phosphorylation levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

In recent years FOXO family members have emerged as key players in controlling cell cycle. FOXO is known to induce cell cycle arrest through p27, a member of the KIP family, which inhibits CDK2 activity and therefore blocks the cell cycle progression at the G1/S-phase. FOXO, as well as GSK-3β, become inactive after phosphorylation. Figure 29 indicates the enhanced levels of phosphorylated FOXO after NOD ligands stimulation.

Fig. 29: FOXO phosphorylation in AtT20 cells. Depicted are the increased phosphorylation levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.



Consequently, I also had a look on the cyclin-dependent kinase inhibitors p27 and p21, respectively. Both inhibitors fulfill their tasks in the late G1/S-phase by inhibiting the CDK2 activity. After all, it was not astonishing that their quantity was reduced. Western Blot analysis discovered low levels of p21 upon stimulation with the ligands for 24 hours (Fig. 30).

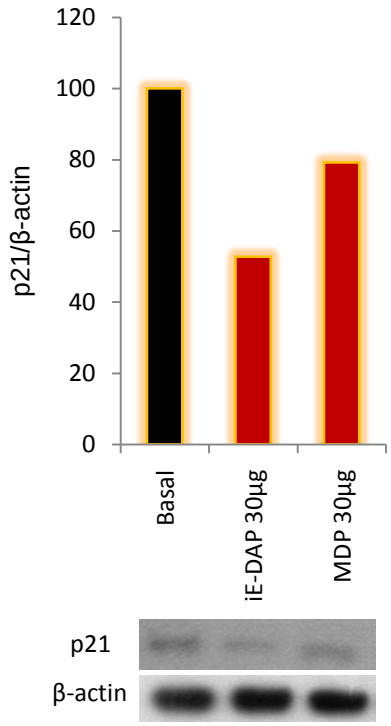


Fig. 30: Downregulation of cyclin-dependent kinase inhibitor p21 in AtT20 cells. Depicted are the reduced protein levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

In the case of p27, overexpression of NOD2 was additionally included in the experiment and mRNA levels of p27 were established by RT-PCR (Fig. 31).

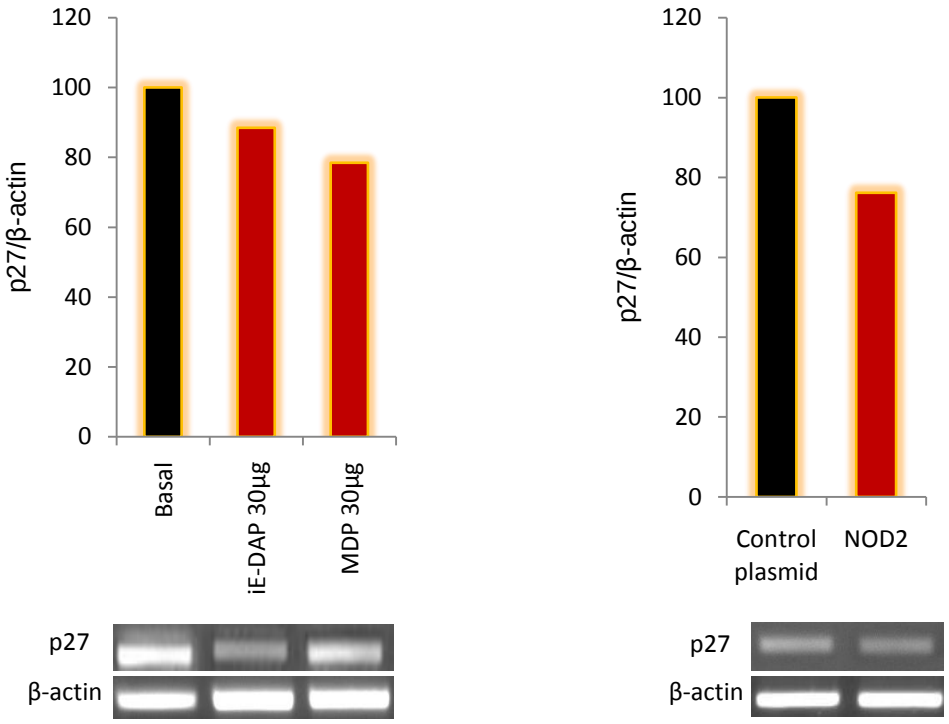


Fig. 31: Downregulation of cyclin-dependent kinase inhibitor p27 in AtT20 cells. Depicted are the reduced mRNA levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours and after overexpression of NOD2. RT-PCR bands were converted in numeric values by ImageJ and are presented in percentage of control.

Taken together, this data support the theory that NOD receptors have a strong influence on cell cycle progression, especially on cell cycle components regulating the G1/S-phase transition. They target mechanisms of cell proliferation and cell cycle, and this could play a role in tumor transformation and survival of pituitary cells.

4.5 A series of biochemical apoptosis events are negatively regulated by NOD receptors

Apoptosis is a fundamental biological process of all multicellular organisms and plays an important role to the cells` fate. The mechanisms that direct a cellular decision whether to live or to die are complex and tightly regulated by abundance of molecules with distinct roles in the signaling process. Here it is considered how NOD receptors affect this process.

The tumor suppressor p53 acts as a guardian, preventing cells from malignant transformation by responding to a variety of signals and triggering cell cycle arrest, apoptosis and senescence. Inactivation of p53 occurs in over half of all human tumors, underscoring the importance of this gene to the pathogenesis of cancer [134]. The function of p53 relies on its ability to induce expression of pro-apoptotic genes including BAX, or to act as an inhibitor of cell cycle by inducing p21 mediated cell cycle arrest [135]. I have already described the effects of NOD1/NOD2 on p21 protein expression. Thus, this chapter will focus on the apoptotic effects of p53. To determine whether there is an involvement of NOD receptors in modulating the transcriptional activity of p53, the luciferase reporter gene assay was performed as described in chapter 3.5.2. AtT20 cells were stimulated with 30 µg/ml of iE-DAP and MDP for 24 hours. The experiment revealed a significant inhibition of p53 luciferase activity (Fig. 32).

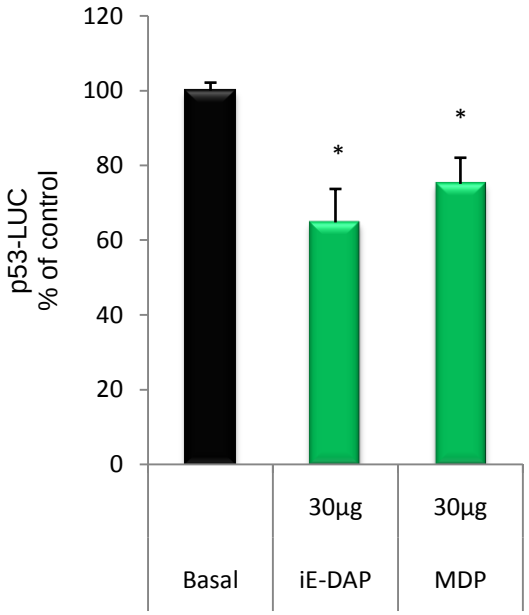


Fig. 32: NOD ligands decrease p53 luciferase activity in AtT20 cells. AtT20 cells were transfected with p53-LUC plasmid and stimulated with 30 µg/ml of iE-DAP and MDP. After 24 hours, luciferase activity was measured. Values are presented in percentage of control and as the mean ± SE (* $P < 0.05$).

The cells were additionally transfected with control plasmid and NOD2 overexpression plasmid and stimulated with 30 µg/ml of MDP for 24 hours. Matching the data with the stimulus, the overexpression of NOD2 also decreased the transcriptional activity of p53 (Fig. 33).

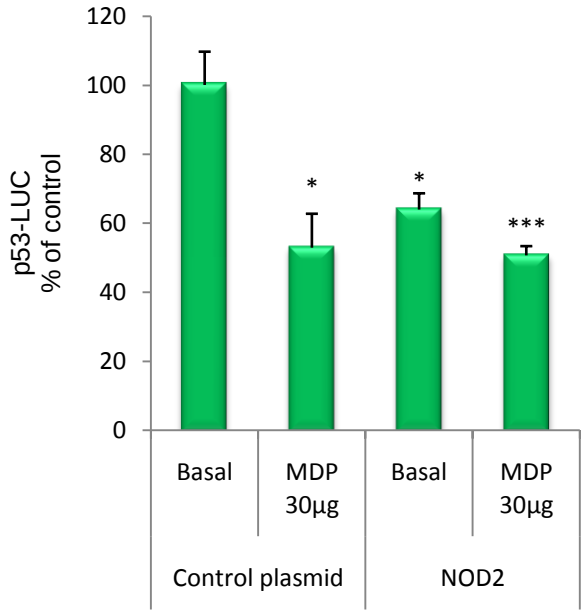


Fig. 33: Decreased p53 luciferase activity in NOD2 overexpressed AtT20 cells. AtT20 cells were transfected with control plasmids or NOD2 expression vector along with p53-LUC with or without 30µg/ml of MDP. 24 hours post-transfection, luciferase activity was measured. Values are presented in percentage of control and as the mean ± SE (* $P < 0.05$ and *** $P < 0.001$).

It is already well known that BAX is a main downstream target of p53. For this reason the connection between p53 and BAX was evaluated. BAX functions as a pro-apoptotic protein and belongs to the BCL-2 gene family [136]. After stimulation of AtT20 cells for 24 hours with NOD ligands and overexpression of NOD2, the protein expression of BAX was investigated. Figure 34 illustrates the reduced protein expression of BAX, assessed by Western Blot.

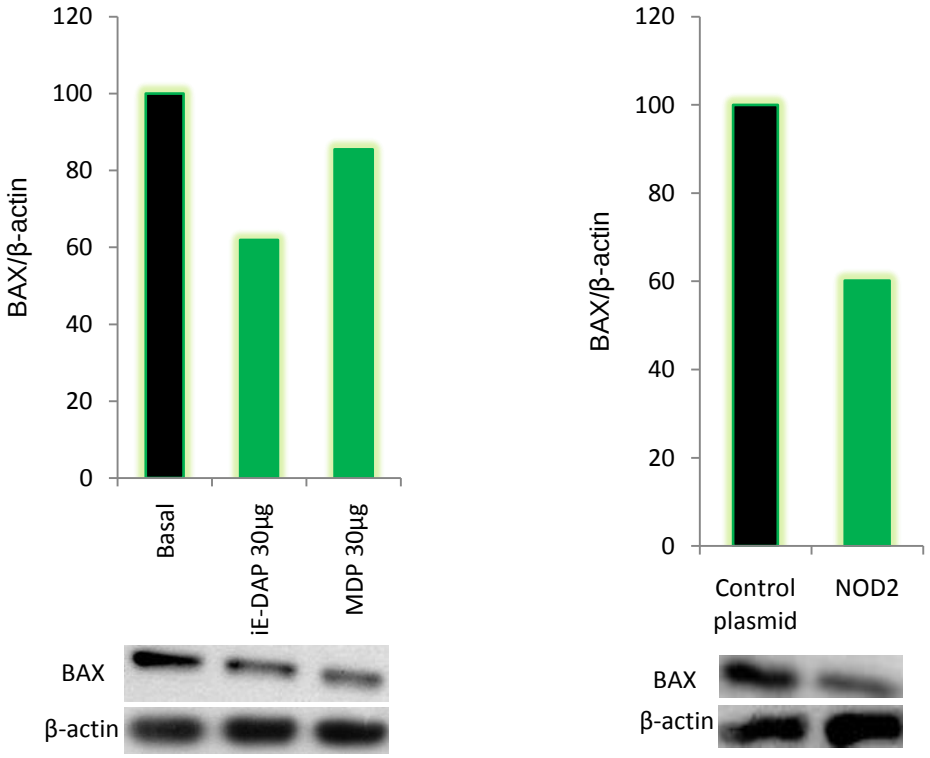


Fig. 34: Downregulation of BAX protein expression in AtT20 cells. Depicted are the reduced protein levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours and after overexpression of NOD2. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

PTEN, another important tumor suppressor is frequently mutated or deleted in several types of cancers and known as a further guardian for a cell to not take the path to cancer [67, 68]. Recently a mechanistic link between PTEN and p53 has been established. As PTEN is frequently mutated in tumors and is able to anagonize the PI3K signaling, the phosphorylation level of PTEN was measured by Western Blot after stimulation with 30 µg/ml of iE-DAP and MDP for 24 hours. An obvious inhibition could be demonstrated (Fig. 35).

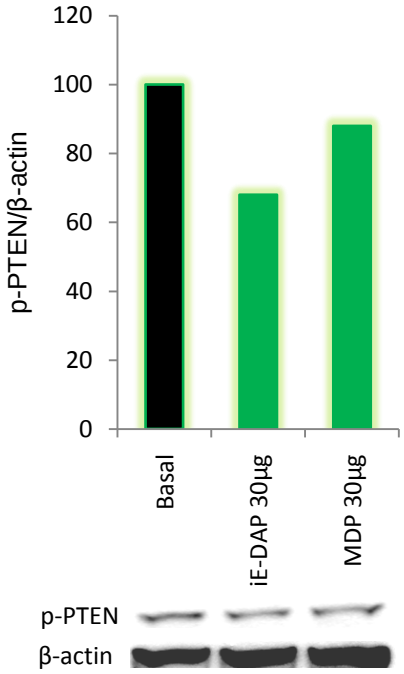


Fig. 35: Downregulation of phosphorylated PTEN in AtT20 cells. Shown are the reduced phosphorylation levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

The XIAP (X-linked Inhibitor of Apoptosis Protein) protein is a member of the inhibitor of apoptosis family of proteins (IAP) and able to stop apoptotic cell death [137]. AtT20 cells were stimulated with 30 µl/mg of iE-DAP and MDP for 24 hours. Proteins were extracted and Western Blot analysis performed (Fig. 36).

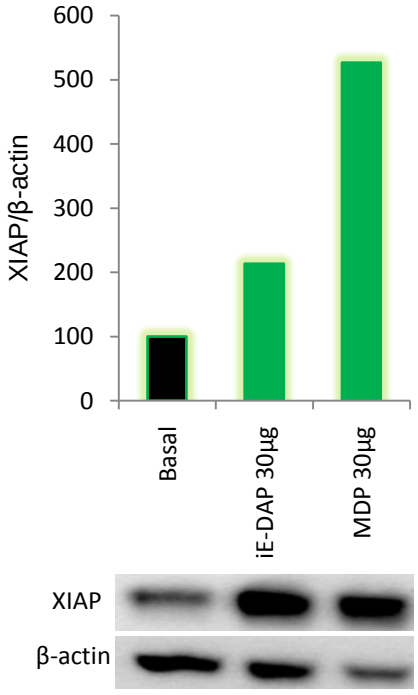


Fig. 36: Upregulation of XIAP protein expression in AtT20 cells. Illustrated are the increased protein levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

The JNK (c-Jun N-terminal Kinase) protein and the AP-1 (activator protein 1) transcription factor are also linked to apoptosis. JNKs mostly target transcription factors and one of these factors is AP-1. Thus, the phosphorylation status of JNK was analyzed using the Western Blot technique. AtT20 cells were stimulated in time-dependent manner with 30 µl/mg of iE-DAP and MDP. The JNK phosphorylation levels were strikingly decreased when compared to basal levels (Fig. 37).

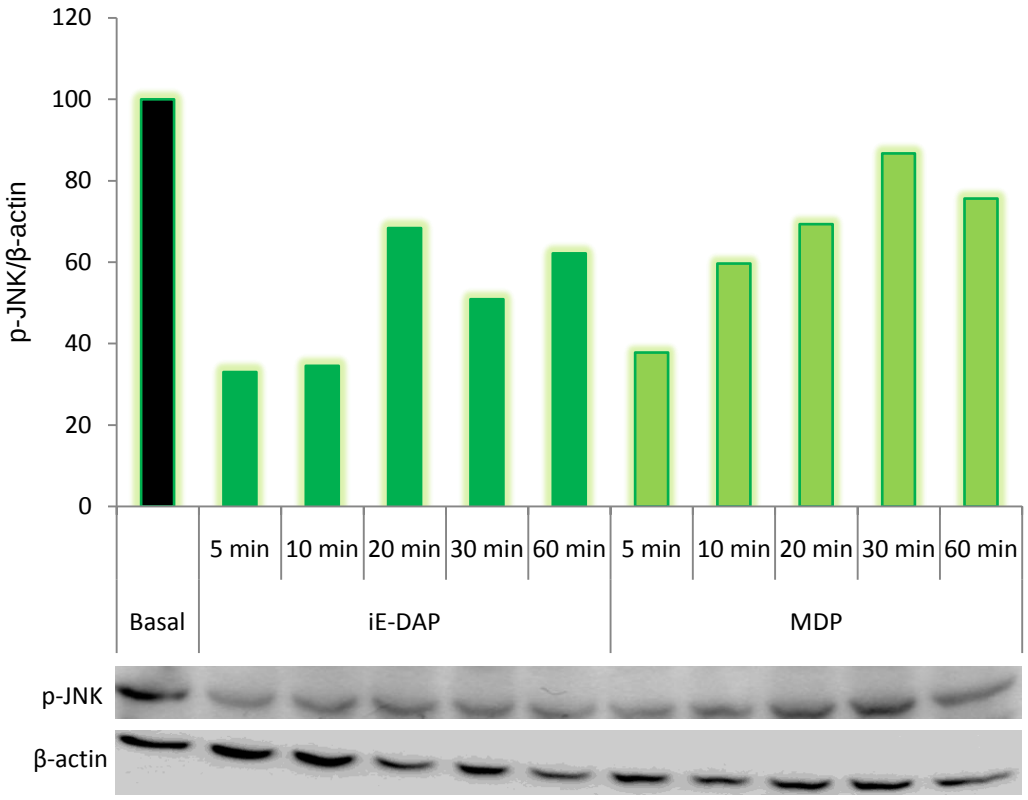


Fig. 37: Downregulation of JNK phosphorylation in AtT20 cells. Illustrated are the decreased phosphorylation levels in time-dependent manner after treatment with 30 µg/ml of iE-DAP and MDP. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

AP-1 is a transcription factor, which regulates gene expression in response to a variety of stimuli and controls a range of cellular processes including apoptosis [138]. The transcriptional activity of AP-1, measured by luciferase gene reporter assay, was significantly inhibited (Fig. 38).

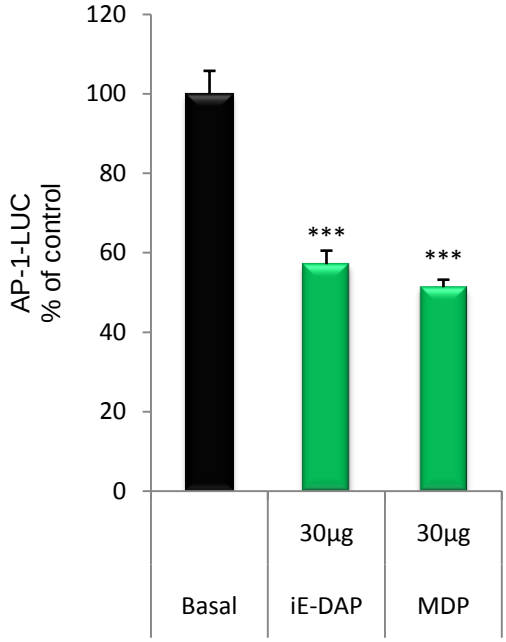


Fig. 38: NOD ligands decrease AP-1 luciferase activity in AtT20 cells. AtT20 cells were transfected with AP-1-LUC plasmid and stimulated with 30 µg/ml of iE-DAP and MDP. After 24 hours, luciferase activity was measured. Values are presented in percentage of control and as the mean ± SE (*** $P < 0.001$).

Taken together, the results presented in this chapter suggest that the AtT20 pituitary cells are driven towards expansion and survival, rather than to apoptosis. The results clearly demonstrate that NOD receptors don`t induce cell death but might support cancer development.

4.6 NOD1 and NOD2 signaling targets related to survival

As described in the introduction, there are many signaling pathways affected by NOD receptors. The most investigated pathway in response to NOD ligands is the NF-κB pathway. This evolutionary conserved pathway is of major importance due to the fact that NF-κB regulates many biological processes including survival. The pathway is also known to act contrary to apoptosis. The PI3K pathway members and the MAPK members, in particular the ERK module and the P38 group, represent further pathways critical to many cellular events and are also mostly associated with survival and proliferation. Therefore, this last chapter of the results part will focus on the pathways related to survival and how they are affected by NOD receptor activation.

4.6.1 NOD receptor activation induce NF-κB upregulation

AtT20 cells were transfected with κB-LUC plasmid and stimulated with 30 µg/ml of iE-DAP and MDP. 6 hours post-transfection, luciferase activity was measured. Both NOD ligands significantly induced NF-κB activation (Fig. 39).

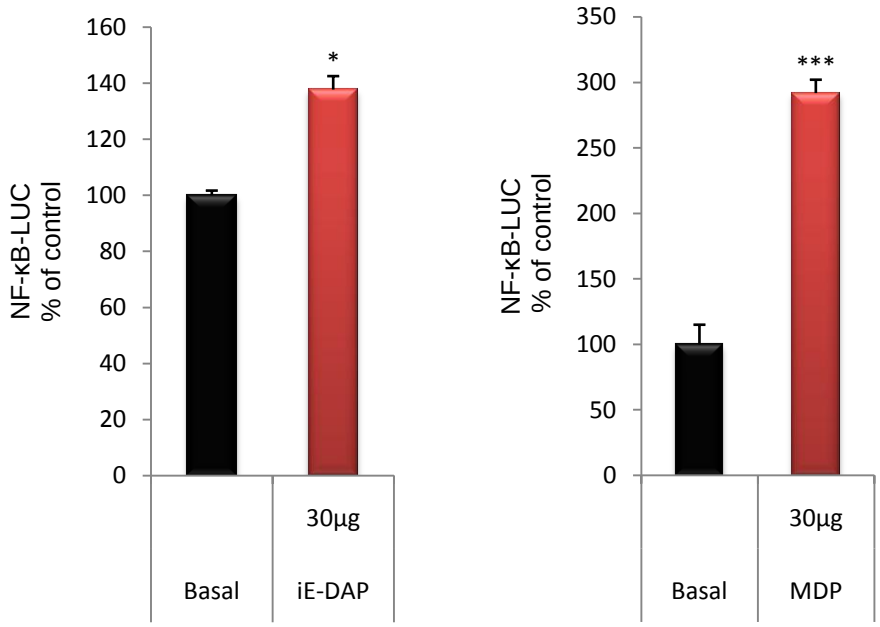


Fig. 39: NF-κB is upregulated by NOD receptors in AtT20 cells. AtT20 cells were transfected and stimulated for 6 hours with κB-LUC plasmid and 30 µg/ml of iE-DAP and MDP. Luciferase activity measurement was followed. Values are presented in percentage of control and as the mean ± SE (* $P < 0.05$ and *** $P < 0.001$).

By protein extraction and Western Blot analysis the phosphorylation levels of NF-κB have been investigated as well. AtT20 cells were stimulated for 5 minutes, 10 minutes, 20 minutes, 30 minutes and 60 minutes with 30 µg/ml of iE-DAP and MDP to verify the phosphorylation status. At each point of this time curve the NOD ligands increased NF-κB phosphorylation (Fig. 40).

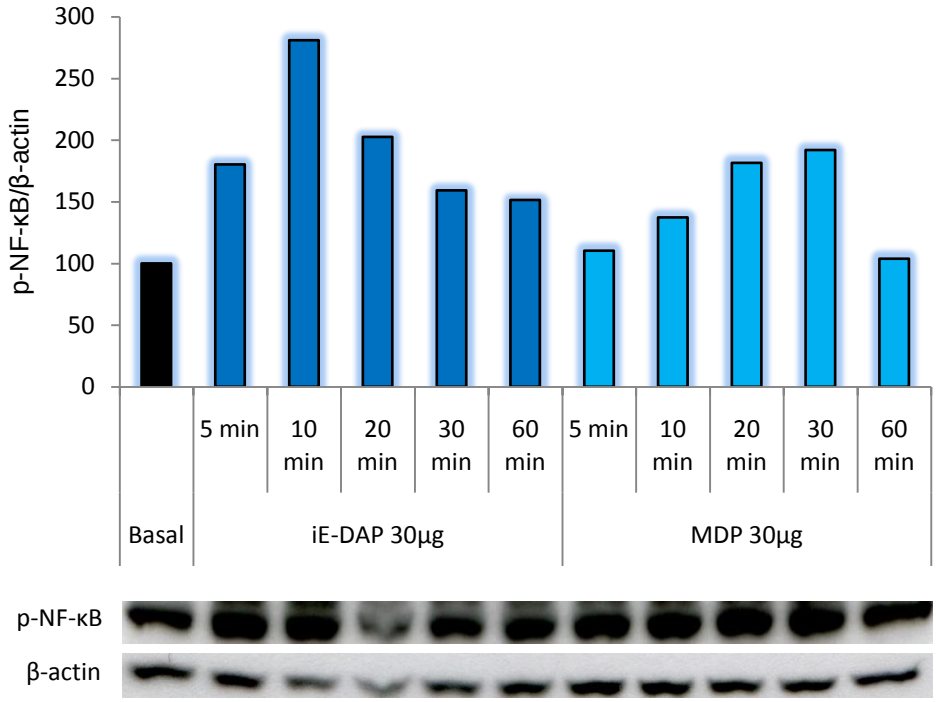


Fig. 40: NF-κB phosphorylation by NOD receptors. Illustrated are the increased phosphorylation levels in time-dependent manner after treatment with 30 µg/ml of iE-DAP and MDP. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

BCL-2 is the prototype of a family of proteins, which can be either pro-apoptotic or anti-apoptotic. It is already shown that NF-κB transcriptionally regulates BCL-2 and that increased levels of BCL-2 make cancer cells more resistant to apoptotic effects [139]. In this experiment only the effect of the NOD2 overexpression was evaluated. AtT20 cells were transfected with the NOD2 overexpression plasmid for 24 hours followed by the protein extraction and Western Blot analysis. The protein expression was elevated in samples transfected with the overexpression plasmid NOD2 (Fig. 41).

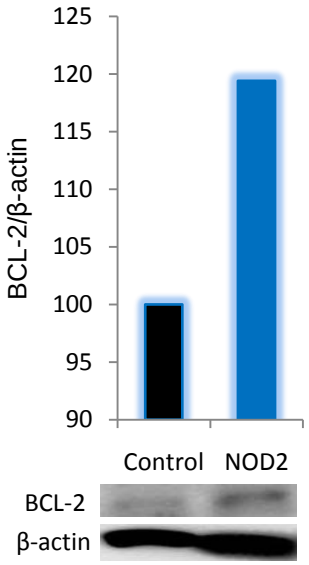


Fig. 41: Upregulation of BCL-2 by NOD2 overexpression. AtT20 cells were transfected with control and NOD2 overexpression plasmid, and proteins were extracted 24 hours post-transfection. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

Furthermore, after transfecting the AtT20 cells with specific siRNA targeting NOD2 and a non-targeting siRNA (Si Control) for 24 hours, the upregulation of BCL-2 could be reversed, proving the specificity of the experiments (Fig. 42).

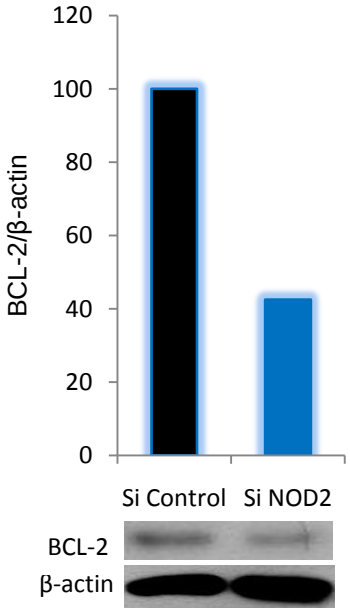


Fig. 42: Downregulation of BCL-2 protein expression by silencing NOD2 in AtT20 cells. Cells were transfected with siRNA for NOD2 and non-targeting siRNA, Si Control. 24 hours post-transfection, proteins were extracted. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

4.6.2 Phosphorylation of PI3K pathway members induced by NODs

As already mentioned, the PI3K pathway and its members play a crucial regulatory role in many cellular processes. As till now the results of the study show increased proliferation and support the idea that the whole system is pushed towards survival, it is interesting to evaluate the contribution of PI3K pathway and its members. AtT20 cells were stimulated and harvested after various periods to observe the changes in the phosphorylation status of AKT. As shown in figure 43 the NOD ligands increased AKT phosphorylation on the Ser473 residue at all times.

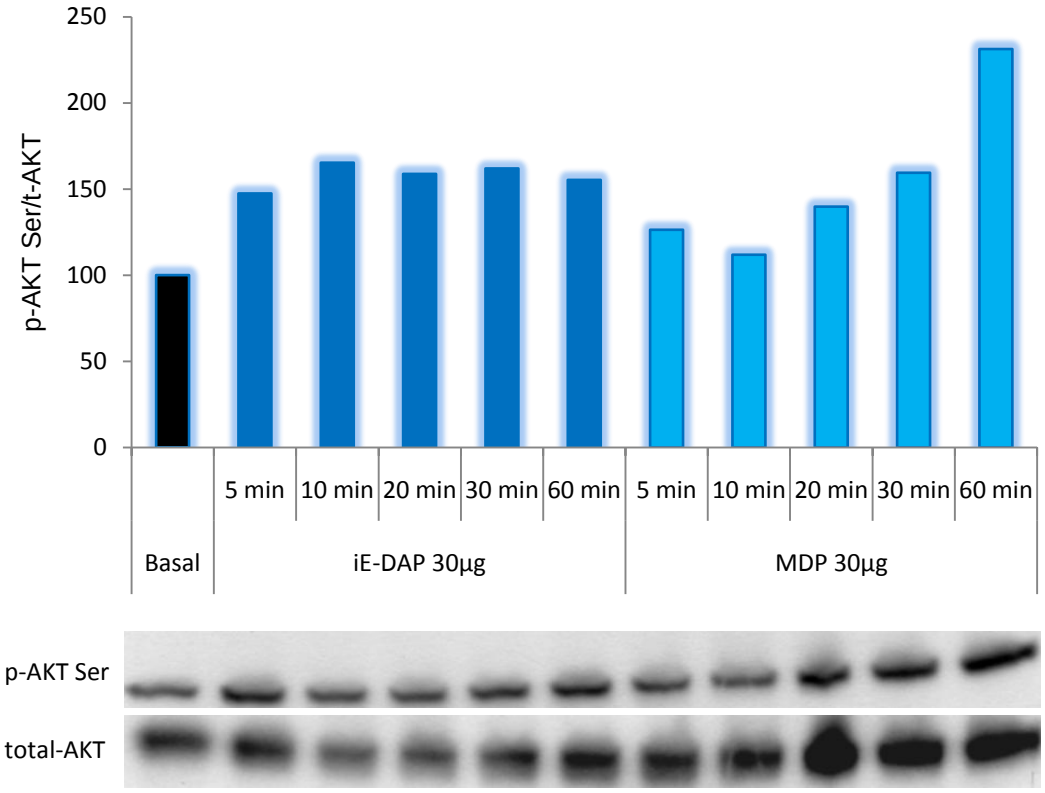


Fig. 43: Phosphorylation levels of AKT Ser473 by NOD receptors. Illustrated are the increased phosphorylation levels in time-dependent manner after treatment with 30 µg/ml of iE-DAP and MDP. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

Specific siRNA against NOD1 and NOD2 as well as a non-targeting siRNA as a control were used to knock down the gene expression of NOD receptors. After transfecting AtT20 cells with siRNA for NOD1 and NOD2 and si Control, the proteins were extracted and the phosphorylation state of AKT was carried out by Western Blot analysis. Interestingly, knock down of NOD1 and NOD2 decreased phosphorylation of AKT on both, Ser473 and Thr308 residues, when compared to si Control (Fig. 44 a +b).

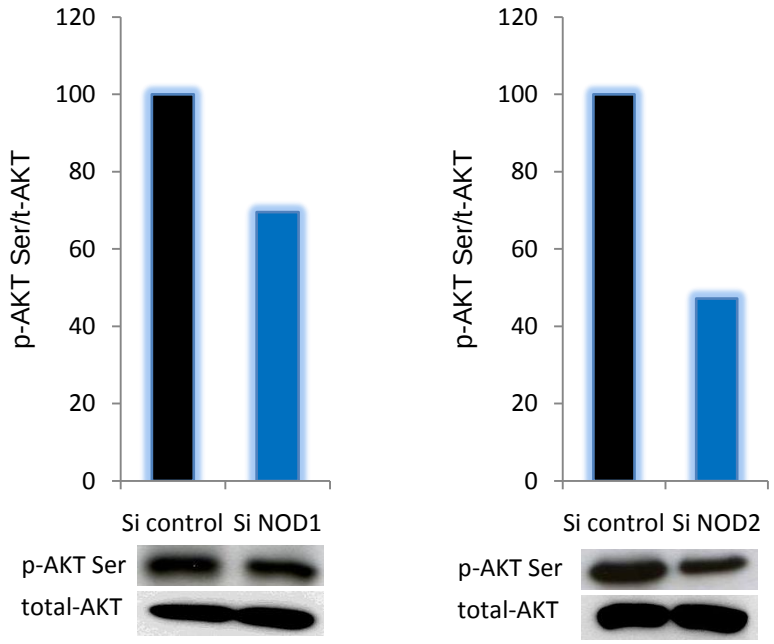


Fig. 44 a: Inhibition of AKT Ser473 phosphorylation by silencing NOD1 and NOD2 in AtT20 cells. Cells were transfected with siRNA for NOD1, NOD2 and non-targeting siRNA, Si Control. The proteins were extracted 24 hours post-transfection. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

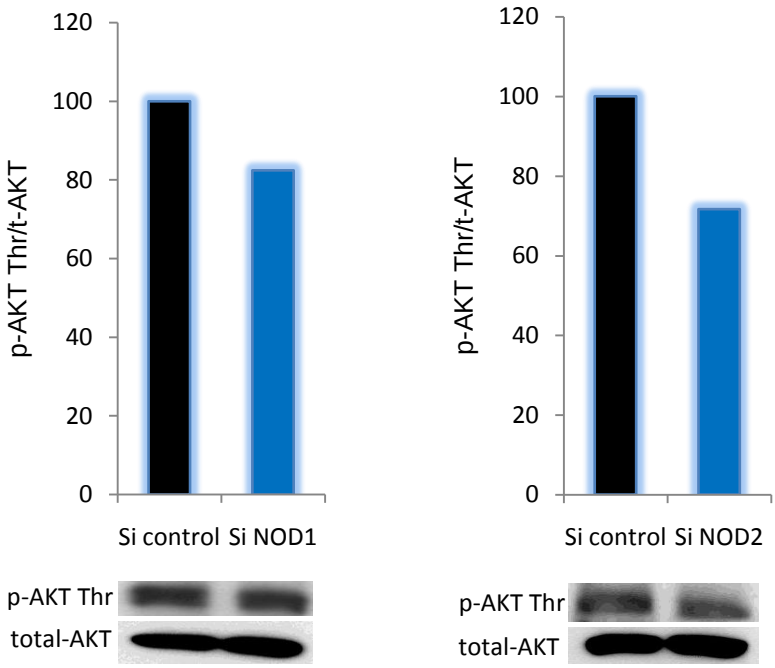
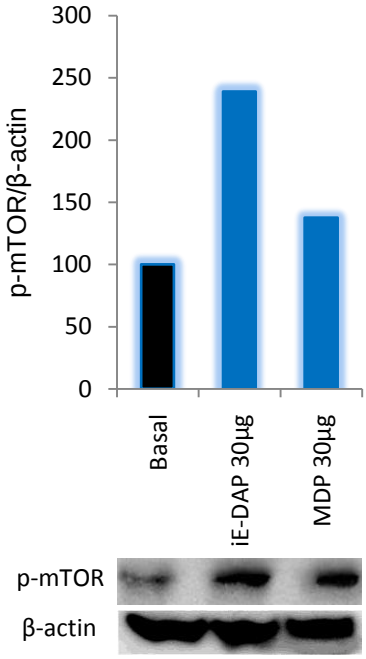


Fig. 44 b: Inhibition of AKT Thr308 phosphorylation by silencing NOD1 and NOD2 in AtT20 cells. Cells were transfected with siRNA for NOD1, NOD2 and non-targeting siRNA, Si Control. The proteins were extracted 24 hours post-transfection. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

Another downstream component of the PI3K/AKT pathway is the serine-threonine kinase mTOR, a key regulator of cell growth and survival [118]. NOD receptor activation by the ligands markedly induced the phosphorylation at residue Ser2448 (Fig. 45).

Fig. 45: Phosphorylation of mTOR at Ser2448 residue in AtT20 cells. Depicted are the increased phosphorylation levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.



Increased levels of mTOR led to an enhanced expression surrounding Ser792 of Raptor in the mTORC1 protein complex (Fig. 46).

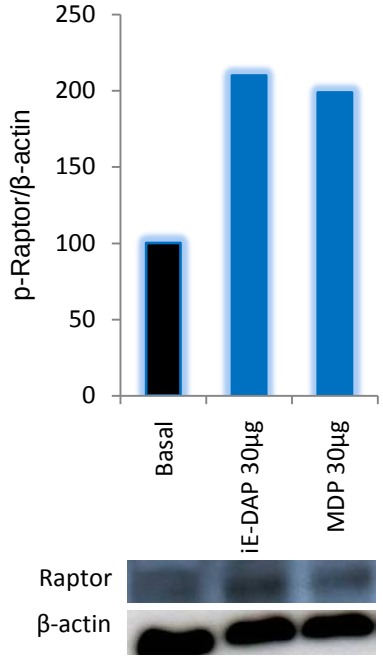


Fig. 46: Phosphorylation of Raptor at Ser792 residue in AtT20 cells. Depicted are the increased phosphorylation levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

The subsequent event of the mTORC1 complex activation is the phosphorylation of its substrate, the p70S6K (p70 ribosomal protein S6 kinase) at the Thr389 residue (Fig. 47).

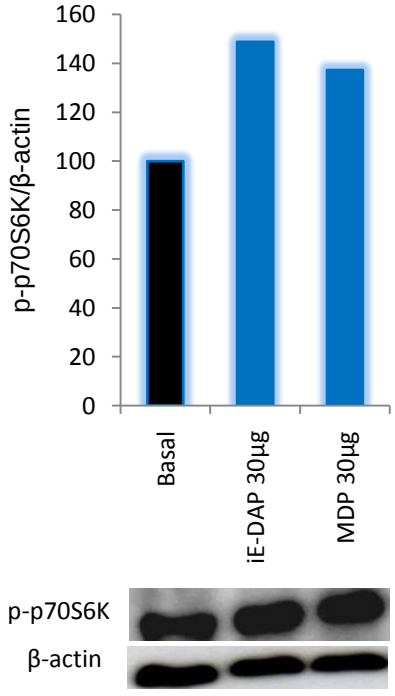


Fig. 47: Phosphorylation of p70S6K at Thr389 residue in AtT20 cells. Depicted are the increased phosphorylation levels after treatment with 30 µg/ml of iE-DAP and MDP for 24 hours. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

4.6.3 NOD receptor activation affects members of the MAPKs

To determine whether NOD receptors affect MAPK pathways, the activation state of the ERK1/2 module and P38 MAPK was investigated. The phosphorylation levels were evaluated after treatment with 30 µg/ml of iE-DAP and MDP at different times. NOD ligands significantly increased phosphorylation of ERK1/2 when compared to unstimulated basal levels (Fig. 48).

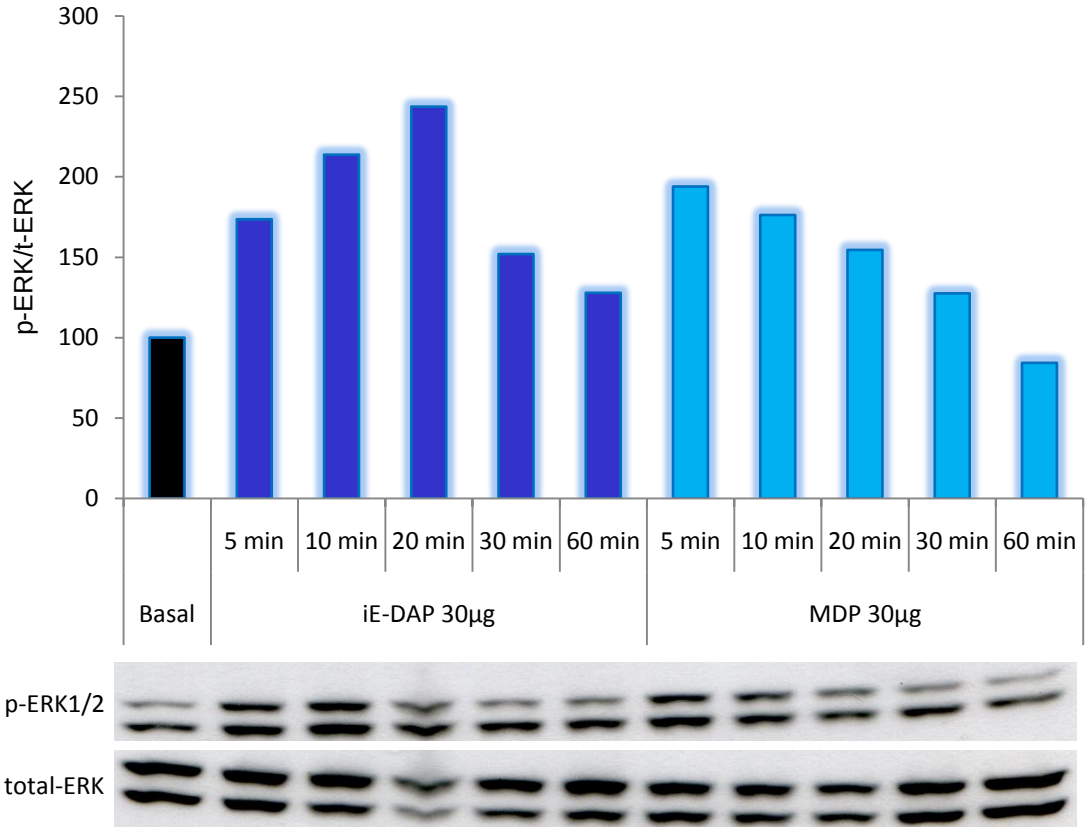


Fig. 48: Increased phosphorylation of ERK1/2 by NOD receptors. Illustrated are the increased phosphorylation levels in time-dependent manner after treatment with 30 µg/ml of iE-DAP and MDP. The upper band shows the ERK1 (p44) protein and the lower band shows the ERK2 (p42) protein. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

Just as the ERK1/2, the phosphorylation state of P38 MAPK showed exactly the same pattern, which means that NOD receptors activation induced phosphorylation of P38 significantly (Fig. 49).

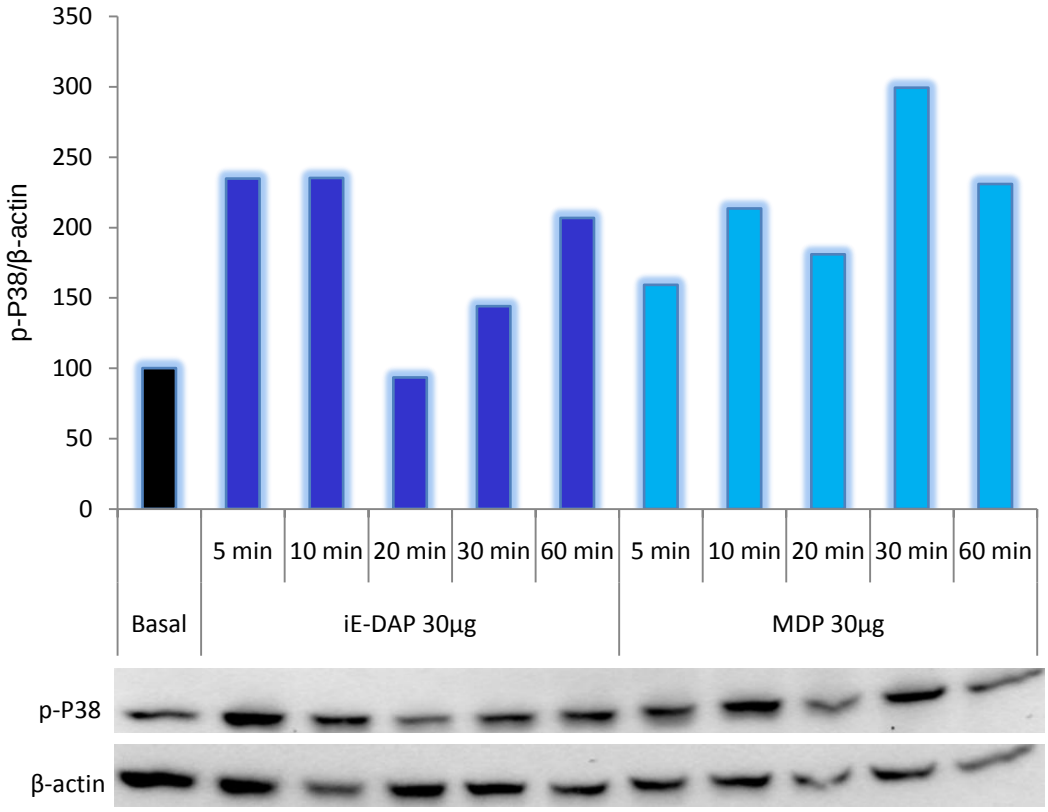


Fig. 49: Increased phosphorylation of P38 by NOD receptors. Illustrated are the increased phosphorylation levels in time-dependent manner after treatment with 30 µg/ml of iE-DAP and MDP. Western Blot bands were converted in numeric values by ImageJ and are presented in percentage of control.

Altogether the outcomes of this last chapter clearly show that, besides the activation of NF-κB, the PI3K/AKT pathway is also affected by NOD receptors activation and is definitely an important downstream target of these molecules. Moreover the findings suggest that NOD signaling may also modulate MAPK levels. All in all the results can be connected to increased proliferation and survival of pituitary tumoral cells AtT20.

5. Discussion

In terms of pathophysiology of the pituitary gland, epidemiologic analyses have identified a much higher incidence of pituitary tumors than previously thought. Pituitary tumors are reported to account 10% - 15% of all intracranial tumors. Many pituitary tumors are silent and not frequently diagnosed, while others may be life-threatening. These tumors cause compression of intracranial structures and hormone hypersecretion. Little is known about the precise environmental and genetic factors leading to their development, so preventive measures are unavailable.

The known physiological significance of NOD receptors lies primarily in their roles in host defense against microbial infections. Many inflammatory and non-inflammatory disease processes can be attributed to dysregulated NOD receptor signaling. Dysreglated NOD2 signaling can lead to either gain of function (constitutive NF-κB activation) or loss of function (defective NF-κB activation) phenotypes. While gain of function mutations have been described in Blau syndrome, loss of function mutations have been associated with Crohn`s Disease [140-141]. In addition, abnormalities in NOD1 function might lead to the development of chronic infection with Helicobacter pylori and to the subsequent development of peptic-ulcer disease and gastric carcinoma [142]. Chronic inflammation plays a multifaceted role in carcinogenesis. Mounting evidence suggests that persistent inflammation functions as a driving force on the way to cancer. Approximately 25% of all cancers are somehow associated with chronic infection and inflammation [143]. As mentioned in the introduction, there is already strong evidence that these two intracellular immune receptors are involved in tumorigenesis. NOD1 signaling pathway regulates inflammation-mediated colon cancer and tumor growth in breast cancer, whereas NOD2 receptors are involved in promoting growth and survival of human colonic carcinomas [76, 77, 78]. The possible mechanisms, by which inflammation can contribute to tumorigenesis imply a distinct network of intracellular signaling molecules, including upstream kinases and transcription factors, which facilitate tumor promotion and progression by increasing proliferation, survival and growth and inducing resistance to apoptosis.

5.1 Expression of NOD immune receptors in the pituitary gland

The starting point of the whole thesis and the basis for all following experiments was the demonstration that NOD immune receptors are expressed in the pituitary gland and ACTH secreting cell line AtT20, which has been used as a model. Immunohistochemistry and RT-PCR experiments, carried out as part of my diploma thesis, clearly revealed the expression of both NOD receptors in AtT20 cells. Moreover, the expression of NOD1 and NOD2 was also detected in normal human pituitaries. These findings suggest that the intracellular immune receptors NOD1 and NOD2 are functional in the pituitary gland. To explore the possible correlation between the presence of pituitary adenomas and the expression of these two intracellular receptors NOD1 and NOD2, samples of four normal pituitaries and thirty-eight different human pituitary adenomas were investigated by using the RT-PCR method. Interestingly, in a considerable proportion of the pituitary adenomas studied, increased mRNA levels of NOD1 and NOD2 were observed, when compared to normal pituitaries. Hence, at that point it could be speculated that NOD expression is involved in pituitary tumor progression.

5.2 Effects on hormone production

After the first evidence of functional NOD receptors in the pituitary gland the question raised, whether there was an impact on hormone production. The AtT20 murine corticotroph cell line is one of the most studied pituitary cell lines and is widely used as a model to understand the physiology and pathology of corticotrophs` action. POMC (Proopiomelanocortin) is synthesized by corticotroph cells and processed to ACTH, which stimulates the adrenal cortex. More specifically, it stimulates the secretion of glucocorticoids. The glucocorticoids in turn exert a classical negative feedback on the Hypothalamic-Pituitary-Adrenal (HPA) axis. Disturbance of this system causes various endocrine syndromes. One of the most severe is the Cushing`s Syndrome, which results from chronic exposure to glucocorticoids. A Cushing`s Syndrome develops after transformation of normal corticotroph cells of the anterior pituitary lobe of a ACTH-secreting pituitary tumor. The transformed cells grow and develop into an ACTH-secreting adenoma. At the moment there is no effective pharmacological therapy to control ACTH over-secretion by pituitary tumors. Positive and negative regulation of the POMC gene has been described and shown to be mediated by several transcription

factors, such as AP-1, NF-κB and Nur elements [144-146]. POMC transcriptional activity was lowered in AtT20 cells after stimulation with NOD1 ligand, iE-DAP, and NOD2 ligand, MDP, as well as by NOD2 overexpression. Corresponding to this inhibition was the elevated activation of NF-κB, induced by NOD receptors. This result fits with the recent discovered mechanism, by which an inhibition of NF-κB DNA-binding activity is required for the transcriptional activation of POMC gene by CRH. Thus, the increased activity of NF-κB is associated with decreased transcriptional activity of the POMC gene. Furthermore, it has been well shown that POMC gene is associated with activation of the AP-1 transcription factor [147]. Two Nur DNA binding sites have been identified on the POMC promoter. The distal Nur response element is NurRE and plays a major role in mediating stimulation through CRH [125]. Both transcription factors, AP-1 and NurRE, were downregulated in AtT20 cells, either by NOD ligands or by NOD2 overexpression. NOD ligands are involved in the regulation of POMC through a mechanism implying other transcription factors. Consequently, ACTH production was low after treatment with the ligands of NOD1 and NOD2 in AtT20 cells. The question arose, whether the NOD receptors also influence hormone production in primary cell cultures of normal rat pituitaries. The idea suggests itself, that the effect of NOD ligands seen in AtT20 cells also takes place in normal rat pituitaries. The next logical step was to evaluate the ACTH levels in normal rat pituitary cells treated with NOD ligands. As the normal rat pituitaries showed no changes in ACTH levels upon iE-DAP and MDP treatment, it could be speculated that the NOD ligands target the pathophysiological side by affecting just tumoral cells, at least in terms of ACTH secretion. This suggests that NOD receptors could be a possible pharmaceutical target.

5.3 Proliferation and Survival

5.3.1 Proliferation

Dysregulation of various cell cycle pathways has been reported to be a frequent event in pituitary tumors [36, 43, 45, 131]. Uncontrolled cellular proliferation is known to consequently lead to tumor development and is in most cases associated with cell cycle dysregulation. Normal mammalian cellular proliferation is monitored at each phase of the cell cycle by a cyclically operating biochemical machinery, constructed from a set of interacting proteins that induce and coordinate proper progression through the cell

cycle. This includes cyclins and cyclin-dependent kinases (CDKs), which promote cell cycle progression, and their inhibitors that oppose cell proliferation. Pituitary adenomas are generally associated with extremely low proliferative rates, especially when compared to AtT20 cells, which are known to be fast reproducing cells.

In AtT20 cells as well as in pituitary adenomas, NOD receptor activation led to increased proliferation levels. The cell proliferation was also assessed by using the PI3K inhibitors, Wortmannin and LY294002, molecules that disrupt the ATP binding pocket of PI3K and PI3K like enzymes. While Wortmannin is an irreversible inhibitor, LY294002 is a competitive inhibitor [127]. The PI3K inhibitors, LY294002 and Wortmannin, as well as the NF-κB inhibitor CAPE (Caffeic acid phenethyl ester) have been used to verify the involvement of these two pathways in the proliferative effect of NOD stimulation. Since all three inhibitors strongly inhibited the mitogenic effect, both pathways may be involved in the proliferation promoting effects of NOD signaling pathways in AtT20 cells. The roles of PI3K/AKT pathway in NF-κB dependent gene expression are controversial. It has been shown that the PI3K/AKT pathway can positively or negatively regulate NF-κB expression [119,148]. In addition, it still remains to be clarified whether NF-κB could act downstream of PI3K/AKT signaling.

NOD receptors activation clearly affects intrinsic mechanisms controlling cell proliferation and the cell cycle which in turn can contribute to neoplastic transformation. Furthermore, NOD receptors signaling may specifically affect the survival of pituitary tumoral cells. Recently it has been reported that in pituitary adenomas both the PI3K/AKT and MAPK pathway are overexpressed, which results in inhibition of cell cycle inhibitors [149-150]. I have shown the increased mRNA expression of NOD receptors in a subset of human pituitary adenomas; they might sustain survival through PI3K/AKT pathway.

There are many factors known that have an influence on CDK4/Cyclin D and CDK2/Cyclin E complexes, which in turn are known to regulate the G1/S transition. The requisite initial steps for cell cycle progression together with the influence of substrates such as pRb (Retinoblastoma) and pGSK-3β (glycogen synthase kinase- 3β), followed by factors involved in cyclin-dependent kinase inhibitors, will be discussed in detail in the following paragraph. After receiving a mitogenic signal, the Cyclin D is the first cyclin produced in the cell cycle and after binding to CDK4, the complex will lead to the initial

phosphorylation of Rb. Western Blot analysis uncovered increased levels of Cyclin D1/D3-CDK4 and Cyclin E-CDK2 in AtT20 cells after treatment with NOD ligands. As I could show increased phosphorylation of ERK 1/2 and P38, and these are mainly associated with proliferation, the initial step of a mitogenic signal is probably initiated by phosporylation of these two MAP kinases. ERK 1/2 is known to be involved in direct control of Cyclin D1 gene expression and P38 increases nuclear CDK2 activity [55, 151]. With respect to cell cycle progression, AKT has been shown to be involved in preventing Cyclin D1 degradation [158]. Thus, Cyclin D expression and stability is increased in different ways.

More recently it has been shown that AKT could negatively influence the expression and localization of cell cycle inhibitors such as p21 and p27 directly or indirectly. This event takes place by blocking FOXO transcription activity and its target, the p27 tumor suppressor gene, or by suppressing p53 activity and its target the p21 tumor suppressor gene [112, 152]. These findings match perfectly to the results obtained in this study, as it has been shown that AKT phosphorylation took place in response to NOD stimulation and its subsequent targets, as there are FOXO and p53, are blocked and are therefore not able to fulfill their duties by inducing p21 and p27 expression [62, 117, 153]. However, in this specific case it still remains to be clarified whether AKT executes this effect directly or indirectly. The connection between AKT and p53 will be discussed in chapter 5.3.2.

The best described function of the Rb substrate is to restrict cell cycle progression within G1-phase. Rb phosphorylation and its subsequent inactivation requires an initial phosphorylation by CDK4/Cyclin D complexes. It has been observed that Cyclin D1 is required for phosphorylation at the Ser780 residue [154-155]. GSK-3β inhibits its kinase activity upon phosphorylation through AKT pathway and subsequent to this event, the degradation of Cyclin D1 will not occur, so that the cell cycle could proceed [55]. As I already observed increased protein levels of Cyclin D1, the activation status of Rb and GSK-3β was investigated. Due to hyperphosphorylation of Rb, free E2F could promote transcription from a number of promoters and several proteins needed for cell cycle progression. The inactivation of GSK-3β also supports the cell cycle progression, as the degradation of Cyclin D1 will not take place.

Figure 50 sums up the preceding paragraphs.

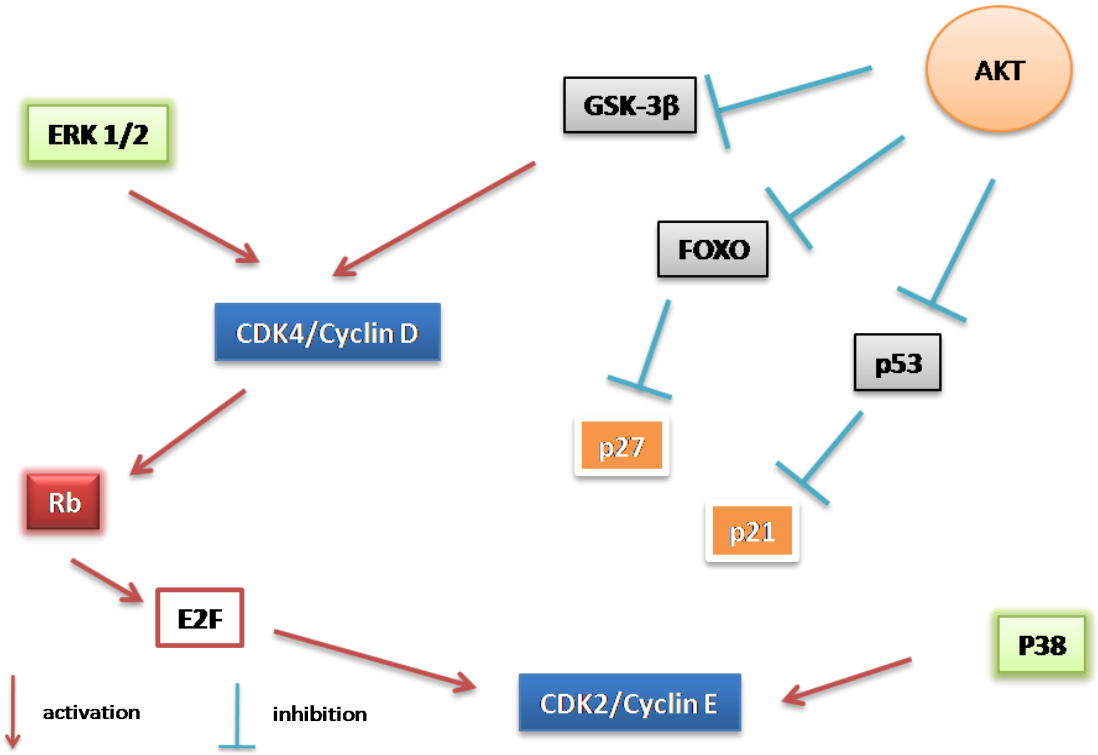


Fig. 50: Illustration of cell cycle progression in AtT20 cells. Initial mitogenic signal by ERK 1/2 and P38. CDK4/Cyclin D complex phosphorylates Rb, free E2F will induce transcription of Cyclin E. AKT phosphorylates and therefore inactivates GSK-3β and FOXO. Moreover, p53 is inhibited by AKT. FOXO and p53 are not able to induce expression of cyclin-dependent kinase inhibitors p27 and p21.

5.3.2 Survival

As several components involved in programmed cell death were negatively regulated by NOD ligands, this section is related to increasing cellular events linked to survival, and decreasing cellular events leading to apoptosis. The first part will describe the involvement of PI3K/AKT signaling pathway and its downstream components. The second part will focus on JNK signaling.

The PI3K (Phosphoinositide 3-kinase) signaling pathway is involved in a large number of cellular functions including proliferation, cell growth and survival. Upon activation, the PI3K leads to the activation of many downstream components. The following part of the discussion will focus on PI3K and its downstream targets as well as on its antagonist, the tumor suppressor PTEN (phosphatase and tensin homolog deleted at chromosome 10). As a primary mediator of PI3K signaling, the role of AKT in supporting tumorigenesis is due to phosphorylation of key regulatory molecules involved in apoptosis, cell growth and proliferation. Related to this study, the tumor suppressor p53, FOXO (forkhead family of transcription factors) and mTOR (mammalian target of rapamycin) have been included [156-157]. The substrates GSK-3β and FOXO are known to be involved in both cell cycle progression and survival, but their main effect is to target cell cycle progression, as has been discussed in the previous chapter.

As I observed increased levels of AKT after treatment with NOD ligands and decreased levels after knocking down NOD1 and NOD2 using the siRNA technique, the specific effect of the receptors could be proven. Thus, the PI3K/AKT signaling pathway seemed to be functional in AtT20 cells upon NOD receptor activation.

PTEN attenuates signaling downstream of activated PI3K and influences many functions, including cell growth, survival, proliferation, migration and metabolism [158]. Western blot analysis revealed low PTEN levels that fit to the elevated AKT levels after NOD ligand treatment. So, the PI3K signaling pathway did not seem to be attenuated by its antagonist. Another important tumor suppressor conntected to the PI3K/AKT pathway is the p53 transcription factor. An impairment of p53 function leads to dysregulation of apoptosis signaling pathways and increases tumorigenesis. NOD receptor activation and NOD2 overexpression led to a suppression of p53. BAX as a representative of the pro-apoptotic part of the BCL-2 family is known as a main target of p53 [136]. In response to apoptotic stimuli, BAX undergoes a conformational change to expose domains vital for its apoptosis function and induces mitochondrial permeabilization [159]. BAX expression was inhibited after treatment with NOD ligands. Interestingly, a connection of PTEN and p53 has recently been established, showing that the absence or mutation of PTEN leads to subsequent loss of the p53 protein and an inability of cells to respond to DNA-damaging agents with an apoptotic response [160].

PI3K/AKT signaling is known to promote translocation of MDM2 (Murine Double Minute 2) from the cytoplasm into the nucleus, where it negatively regulates p53 [117]. AKT is moreover able to induce survival through IKK (I kappaB kinase) and therefore inducing the activation of NF-κB [112]. As has been shown here, p53 was suppressed and activation of NF-κB could be shown on both transcriptional and protein levels. The NF-κB pathway targets genes, that regulate physiological processes ranging from cell proliferation to cell death. A strong correlation has been established between constitutively expressed NF-κB and chronic inflammation, which in turn is a determinant in the development of many cancers [100-101]. BCL-2 is one of the survival representatives of the BCL-2 family and is regulated by NF-κB [139]. Experiments in which NOD2 was overexpressed, suggest that BCL-2 is upregulated. Apart from BCL-2, NF-κB could also induce the expression of XIAP (X-linked Inhibitor of Apoptosis) and as shown in chapter 4.5.2, XIAP protein expression was enhanced after NOD receptor activation. XIAP is known to stop apoptotic cell death by binding and inhibiting caspases 3, 7 and 9, respectively [137]. Paradoxically, a growing body of evidence also exists to support a modulatory role for XIAP in NF-κB activation. Earlier studies revealed the ability of ectopically expressed XIAP to activate NF-κB-responsive reporter activity [161]. That could mean that in the case of survival, expression of XIAP would lead to augmented NF-κB activity and to an amplification of the anti-apoptotic response, besides inhibition of caspases. There is recent evidence that XIAP also promotes the activation of PI3K/AKT pathway and that increased levels of XIAP directly correlate with lowered protein levels of PTEN [162-163].

mTOR (mammalian Target of Rapamycin) is another important downstream target of PI3K/AKT signaling that regulates cell growth and metabolism in response to environmental triggers. The mTORC1 complex is composed of the mTOR catalytical subunit, Raptor, which is the regulatory associated protein of mTOR, PRAS40 and the protein mLST8. One of the most extensively characterized downstream targets of mTORC1 is the ribosomal protein S6 kinase, p70S6K. This kinase is crucial to the regulation of protein synthesis. Thus, activation of mTOR may provide tumor cells with a growth advantage by promoting protein synthesis [118]. As shown in chapter 4.6.2, mTOR, Raptor, as well as p70S6K have been enhanced after treatment with NOD ligands. Even though the results have shown evidence that in AtT20 cells the mTOR signaling pathway is active, the mediated increase in cell mass remains to be shown.

The JNK signaling pathway is known to be required for embryonic morphogenesis and contributes to the regulation of multiple physiological processes. Indeed, the JNK pathway has been implicated in both apoptosis and survival signaling. The specific role of JNK may therefore depend upon the cellular context. In addition it has been reported that JNK also plays an important role in tumor cells. The general consequence of chronic JNK signaling is apoptosis. JNK is known to target the AP-1 transcription factor [164]. The data revealed decreased protein levels of phosphorylated JNK and subsequently decreased AP-1 transcriptional activity. It may be considered as an anti-apoptotic event in this context. However, the precise role of AP-1 in the response of JNK activation is likely to be modified by the activity of other transcription factors that interact with AP-1 on the promoters of target genes. The decision whether AP-1 is oncogenic or anti-oncogenic might depend on tumor type, tumor stage and also the genetic background of the tumors. Although the results on JNK and AP-1 have been considered as an anti-apoptotic event, the question concerning the ability of cells to interpret JNK activation that could lead to different outcomes in different contexts, still remains unresolved and requires more investigation.

The last chapter could be concluded by saying that NOD receptor activation seems to play an important role to the survival of pituitary tumor cells by regulating PI3K/AKT signaling, and as a consequence of this, modulating a variety of AKT downstream targets. Figure 51 represents a general view of the topics discussed in the previous paragraphs.

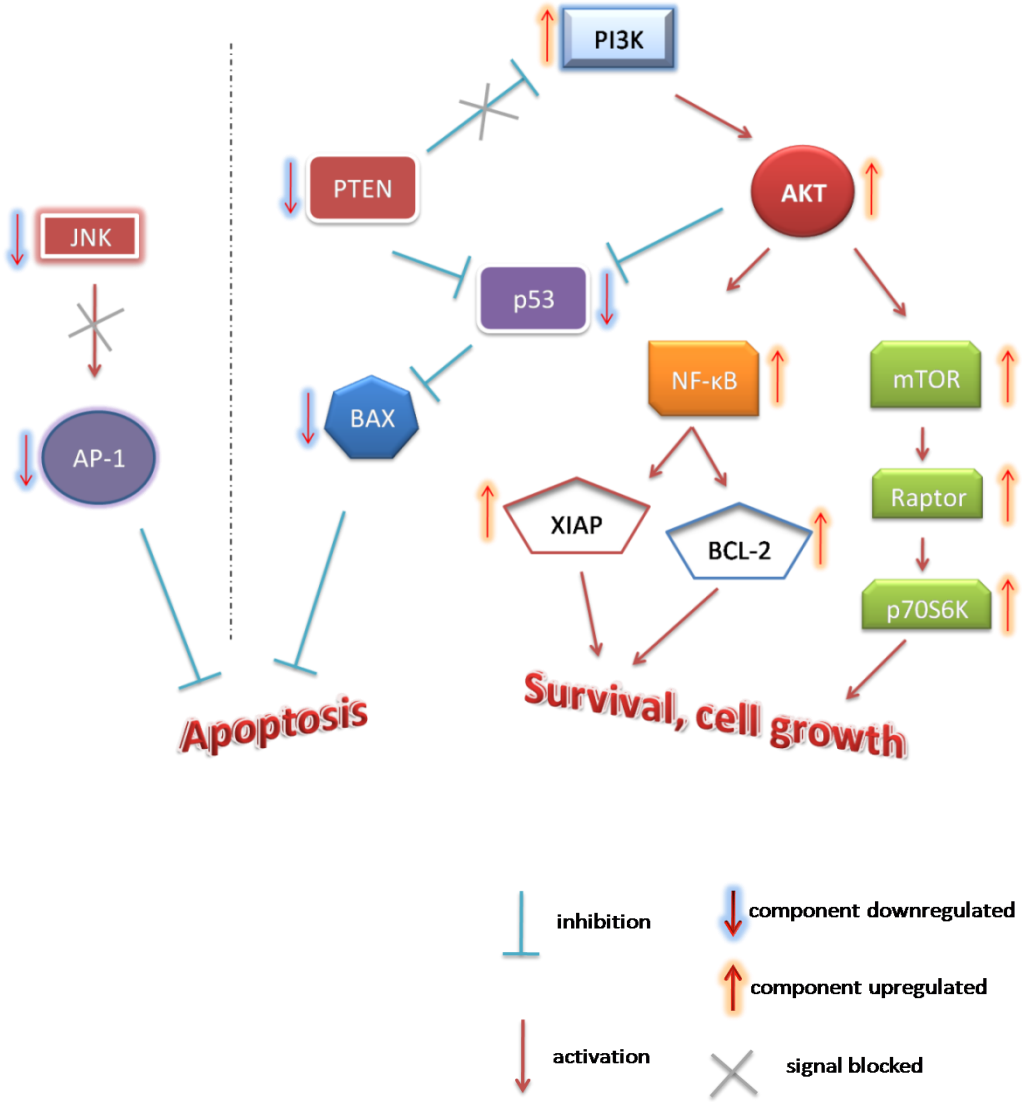


Fig. 51: Illustration of NOD induced cell survival in AtT20 cells. Low levels of PTEN cannot suppress PI3K and lead to loss of p53. Elevated PI3K activates AKT, which in turn activates NF-κB and mTOR. Activated NF-κB will induce pro-survival proteins, XIAP and BCL-2. p53 is inhibited by AKT and therefore the pro-apoptotic BAX protein expression is also reduced, inhibiting apoptotic events. Low levels of phosphorylated JNK could not induce the transcription factor AP-1, thus preventing the cells from cell death.

5.4 Concluding remarks and future perspectives

In summary it can be said, that the results represent a new perspective into infection-associated tumorigenesis, and illustrate the importance of immune receptors in the pituitary gland and possibly in pituitary adenomas. Overall, pituitary adenomas progression may employ the sustained activation of NOD signaling pathways. A

dysregulation of NOD signaling can affect the host-environment interaction and thus contribute to the pathogenesis of this disease.

In the following last paragraphs of the thesis, some future perspectives of NOD receptor signaling are provided. Cells of all organisms transiently or continuously suffer from a variety of damages from internal as well as external physico-chemical and biotic factors during their lifespan. Therefore, the cells have developed several strategies to repair the damage, and if not possible, to eliminate damaged cells. In these processes, so called Heat Shock Proteins (HSPs), produced by stressed cells, play an important role [165]. At least for HSP90 it has been shown that it interferes with NOD receptors [166]. Malignant cells are permanently exposed to stress. HSP can be actively secreted by tumor cells or released by apoptotic tumor cells and can then directly interfere with NOD receptors. From preliminary data performed during this study, HSP90 mRNA levels were higher in pituitary adenomas in comparison with normal human pituitaries. Based on this, it could be hypothesized that HSP proteins act as an endogenous ligand for NOD receptors` mediating effects, and that they act in synergy to influence pituitary tumor development. Thus, blocking the action of bacterial cell wall components or HSP through NOD would then suppress tumor development and may represent a future therapeutic option in the treatment of pituitary tumors. Inhibition could be performed by blocking intracellular signaling pathways and by downregulating components of NOD signaling on transcriptional and translational level.

As NOD ligands seem to not influence normal rat pituitary ACTH secretion, but show effects on ACTH secretion in tumor cells, their contribution to pathogenesis could be emphasized. Thus, NOD receptors could be considered as therapeutic targets; the modulation of NOD receptors activity might slow down or even stop pituitary tumor progression. Here, clearly more work is needed.

Therapeutic antibodies are a fast growing class of human therapeutics. As they provide high specificity, they represent ideal agents for blocking certain processes in tumor development. They can be used to capture or eliminate tumor growth-supporting factors, and therefore to reduce the level of tumorigenic factors. Other antibodies are used to target epitopes on the cell surface involved in receptor-ligand binding. Previously it has been shown that My4, an antibody preventing TLR4/CD14 interaction, blocked LPS induced IL-6 secretion in TtT/GF pituitary tumor cells [167-168]. Recently,

we could show that in TtT/GF pituitary tumor cells, TLR4 and NOD receptors act in a synergistical manner to cause effects [80]. As TLR4 is also expressed in AtT20 tumor cells, neutralizing antibodies might prevent the cells from proliferation and growing. Since no corresponding tools for targeting intracellular NODs exist by now, these effects could only be tested on TLR4 mediated effects, or on effects meditated through the synergy of TLR4 and NODs.

RNA interference is an effective gene-silencing tool for research, as through silencing NOD1 and NOD2 the stimulatory effects on proliferation, survival and growth of pituitary tumor cells could be suppressed. Many difficulties need to be solved until RNA interference will be applicable as a treatment for patients, but they represent promising future cancer therapeutics and may have potential in future clinical treatment concepts [169].

As a chronic bacterial infection through cell wall components may act tumorigenic also in pituitary adenoma, the inflammation-cancer link affirms that targeting the aberrant inflammatory process is an important point in the battle against cancer. Despite extensive research, the pathogenesis of the majority of pituitary adenomas remains to be clarified, as well as the investigation of the most effective way to pharmacologically inhibit proliferation, survival and growth of pituitary tumor cells, especially when related to inflammation induced tumorigenesis.

6. Summary

Although extensive evidence connects the immune system with tumor development and progression, the role of NOD (nucleotide oligomerization domain) receptor signaling in tumor progression and pathophysiology of tumors in general is still poorly understood. This study asks whether NOD receptor activation may play a role in the pathophysiology of the pituitary gland by modulating survival signaling pathways and the activity of genes related to proliferation, survival and death. NOD1 and NOD2 were shown to be expressed in the pituitary gland and in AtT20 cells, and the expression was increased in a considerable proportion of human pituitary adenomas in comparison with normal pituitaries. NOD1 ligand (iE-DAP) and NOD2 ligand (MDP) induced cell proliferation in pituitary adenomas and ACTH secreting cell line AtT20; consistent with this, NOD ligands induced increased levels of G1 CDKs and cyclins. NOD receptor activation also downregulated or inactivated cell cycle regulators (Rb, FOXO and GSK-3β) and cyclin-dependent inhibitors (p21 and p27). This study also showed that NOD ligands activated NF-κB and MAPK signaling, which are known to affect important events to cancer cells. They also activated the PI3K/AKT signaling in AtT20 cells by inducing the phosphorylation of AKT, mTOR, the AKT mammalian target of rapamycin, and p70S6K. In contrast, the downregulation of tumor suppressors was reflected by decreased levels of PTEN (phosphatase and tensin homolog), p27 and p53. Pro-apoptotic molecules, namely BAX, p-JNK and AP-1, were all downregulated, whereas anti-apoptotic molecules, like BCL-2 and XIAP, were upregulated. This study strongly supports the hypothesis that NOD activation promotes the survival of pituitary tumoral cells through different pathways and proteins affecting the fate of a cell. The signaling axis of NOD receptors may promote pituitary cancer progression, but would also be an attractive target for therapy.

7. Zusammenfassung

Obwohl bereits eindeutig bewiesen, dass das Immunsystem mit der Entstehung von Tumorerkrankungen in direkten Zusammenhang gebracht werden kann, ist die Rolle von NOD (nucleotide oligomerization domain) Proteinen bezüglich der Tumorprogression und Pathophysiologie von Tumoren bislang nur mangelhaft beschrieben. Demzufolge wurde in dieser Dissertation überprüft, ob die Aktivierung der intrazellulären NOD-Rezeptoren, und die daraus resultierende Regulation unterschiedlicher Signalwege und Aktivierung von Genen, welche im Zusammenhang mit Proliferation, Überleben und Zelltod stehen, einen kritischen Punkt in der Pathophysiologie von Hypophysentumoren darstellen könnte. Die Expression von NOD1 und NOD2 konnte in der Hypophyse und in AtT20-Zellen nachgewiesen werden. Diese Studie offenbarte eine gesteigerte mRNA-Expression von NOD1 und NOD2 in einem beträchtlichen Anteil an humanen Hypophysenadenome im Vergleich zu normalen humanen Hypophysen. iE-DAP als NOD1-Ligand und MDP als NOD2 Ligand, regten beide die Proliferation in der ACTH produzierenden Zelllinie AtT20 und in humanen Hypophysenadenome an. Die gezeigte Proliferation konnte durch eine sichtlich erhöhte Proteinexpression von G1 Cyclin-abhängigen Kinasen (CDK) und deren Cycline, welches Indikatoren für einen voranschreitenden Zellzyklus darstellen, bekräftigt werden. Im Zuge der NOD-Rezeptoren-Aktivierung wurden einige Regulatoren des Zellzyklus (Rb, FOXO und GSK-3β) und einige Cyclin-abhängige Inhibitoren (p21 und p27) inaktiviert oder herunterreguliert. Diese Studie belegt weiterhin, dass die NOD-Liganden unterschiedliche Signalwege aktivieren, darunter NF-κB and MAP-Kinasen, die dafür bekannt sind, bedeutende Ereignisse in einer Zelle zu beeinflussen. Durch Phosphorylierung von AKT, mTOR und p70S6K aktivierten die NOD-Liganden zusätzlich den PI3K/AKT Signalweg. Im Gegensatz dazu, wurden Tumorsuppressorgene wie PTEN (phosphatase and tensin homolog), p27 und p53 herunterreguliert. Pro-apoptotische Moleküle, wie BAX, p-JNK und AP-1 wurden alle inhibiert, wohingegen anti-apoptotische Moleküle, wie BCL-2 und XIAP, hochreguliert wurden. Durch den Einfluss der NOD-Rezeptoren auf unterschiedliche Signalwege und Proteine, die bedeutend für das Schicksal einer Zelle sind, bekräftigt diese Studie die Hypothese, dass eine NOD-Rezeptor-Aktivierung das Überleben von Hypophysentumorzellen unterstützt. Die NOD-Signalwege können zwar die Weiterentwicklung von Hypophysentumoren unterstützen,

aber gleichzeitig auch einen attraktiven Ansatzpunkt zur Therapie von Hypophysentumoren darstellen.

8. References

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2006	Praktikum bei Micromet, Inc. Department of Immunotherapy – Research & Development • Thema: Generierung eines BiTE-Moleküls
2004 – 2006	Werkstudentin bei Micromet, Inc. o Molekularbiologische Arbeitstechniken: Restriktionsenzymverdau, PCR, Agarosegelelektrophorese, DNA-Aufreinigung, Ligation und Transformation von Plasmiden in *E.coli* o Zellkultur: Transfektionen, Produktion der BiTEs, Kultivierung verschiedener Zellarten, Klonierung von Zelllinien o Durchflußcytometrie (FACS) o Proteinaufreinigung: Affinitätschromatographie (IMAC), Gelfiltrationschromatographie o Proteinanalytik: SDS-Page, Western Blot o Bioassays: ELISA, Zytotoxizitätsassays
2001-2002	Freie Mitarbeiterin im Nachhilfeinstitut Schulfit
2001	Vollzeitbeschäftigung beim DER Business Travel

Publikationen:

Fröhlich B, Labeur M, Paez Pereda M, Buchfelder M, Renner U, Stalla GK, Correa-de-Santana E (in preparation) NOD1 and NOD2 receptors differently affect proliferation and ACTH production in normal and tumoral corticotroph cells.

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