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Deciphering the pathways leading to the enhancement of NIS transcription in thyroid tissue

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RESUMO

O simportador de sódio e iodo (NIS) é expresso na membrana basolateral das células foliculares da tiróide e transporta iodo para o interior destas células. A sua expressão em carcinoma da tiróide permite usar iodo radioativo (RAI) como ferramenta terapêutica. No entanto, uma proporção significativa de tumores perdem a capacidade de captar iodo, devido sobretudo à baixa expressão funcional de NIS, não havendo alternativas terapêuticas eficientes.

O nosso objetivo principal foi avaliar o impacto das vias de sinalização oncogénicas, PI3K/Akt/mTOR e NF-κB, na expressão de NIS. Desta forma, começámos por determinar se a ativação da via canónica de NF-κB, induzida pelo fator TNF-α, afeta a regulação transcricional de NIS. Seguidamente, avaliámos igualmente o efeito da inibição simultânea de PI3K e mTOR.

Este estudo demonstrou que NF-κB desempenha um papel na diminuição da expressão de NIS através da ativação da via com TNF-α e Phorbol-12-miristato-13-acetato (PMA) em células PCCL3. Na linha celular K1, que apresenta uma expressão de NIS muito baixa e na qual a sinalização de PI3K está constitutivamente ativa, conseguimos restaurar a expressão do simportador após a sua inibição.

O estudo das vias de sinalização envolvidas na regulação de NIS, será relevante no desenvolvimento de terapias para o aumento da eficiência captação de iodo em carcinomas da tiróide refratários à terapia com RAI.

Palavras-chave: Simportador de sódio e iodo; Carcinoma da tiróide; Iodo radioativo; PI3K/Akt/mTOR; NF-κB.

ABSTRACT

The sodium-iodide symporter (NIS) is expressed in the basolateral membrane of thyroid follicular cells and transports iodide into its interior. Its expression in thyroid cancers allows the use of radioactive iodine (RAI) as a therapeutic tool. Unfortunately, a significant proportion of patients lose the ability to uptake RAI due mainly to impairment of NIS function, without effective therapeutic alternatives.

Our main purpose was to extend the analysis of the impact of the PI3K/Akt/mTOR and NF-κB oncogenic signalling pathways on NIS expression. In an attempt to achieve that goal we first determined if the activation of the canonical NF-κB pathway, induced by the tumour necrosis factor alpha (TNF-α), affects NIS transcriptional regulation. Next, we also evaluated the effect of simultaneous inhibition of PI3K and mTOR.

This study pointed to a role of NF-κB on the down-regulation of NIS mRNA expression by treatment with TNF-α and phorbol 12-myristate 13-acetate (PMA) in PCCL3 cells. In the K1 cell line, which has very poor expression of NIS and a constitutively active PI3K pathway, we were able to restore NIS expression upon its inhibition.

The extended knowledge of these pathways involved in the regulation of NIS will be relevant for the development of therapies for the enhancement of iodide uptake efficiency in RAI refractory thyroid carcinomas.

Keywords: Sodium iodide symporter; Thyroid carcinoma; Radioactive iodine; PI3K/Akt/mTOR; NF-κB.

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LIST OF ABREVIATIONS, ACRONYMS AND SYMBOLS

Akt- protein kinase B (PKB)

APS- ammonium persulfate

ATC- anaplastic thyroid cancer

ATPase- adenosine 5'-triphosphatase

BAF- B-cell-activating factor

BEZ- BEZ-235

BMS- BMS-345541

BRAF- rapidly accelerated fibrosarcoma type-B

bp- base pair

cAMP- cyclic adenosine monophosphate

C cells- parafollicular cells

CD40- cluster of differentiation 40

cDNA- complementary DNA

CRE- cAMP-responsive element

CREB- cAMP-responsive element binding protein

C terminus- carboxyl terminus

DAPI- 4′,6-Diamidine-2′-phenylindole dihydrochloride

DMEM- dulbecco’s modified eagle medium

DMSO- dimethyl sulfoxide

DNA- deoxyribonucleic acid

DTT- dithiothreitol

dNTP- deoxynucleotides

DPBS- dulbecco's phosphate-buffered saline

DTC- differentiated thyroid cancer

EBV- Epstein-Barr virus

ECL- enhanced chemiluminescence

EDTA- ethylenediamine tetraacetic acid

ERK- extracellular signal-regulated kinase

FBS- fetal bovine serum

FTC- follicular thyroid carcinoma

g- gram

GDP- guanosine diphosphate

GEF- guanine nucleotide exchange factor

G protein- guanine nucleotide-binding proteins

GDP- guanosine diphosphate

GTP- guanosine triphosphate

h-hour

HRP- horseradish peroxidase

I^-- iodide

IKK- IκB kinase

IL-1- interleukin-1

IκB- inhibitor of κB

K^+- potassium ion

KCl- potassium chloride

L-liter

LMP-1- latent membrane protein 1

LPS- lipopolysaccharide

Ltβ- lymphotoxin beta

LY- LY294002

m- meter

M- molar

MAPK- mitogen activated protein kinase

MEK- MAPK/ kinase

$MgCl_2$- Magnesium Chloride

min- minute

mRNA- messenger RNA

MTC- medullary thyroid cancer

mTOR- mammalian target of rapamycin

mTORC1- mTOR complex 1

mTORC2- mTOR complex 2

Na+- sodium ion

NF-κB- nuclear factor kappa-light-chain-enhancer of activated B cells

NIK- NF-κB-inducing kinase

NIS- sodium iodide symporter

NLS- nuclear localization signal

ns- not statistically significant

N terminus- amino-terminus

NUE- NIS upstream enhancer

p- p-value

PAX8- paired box gene 8

PBF- PTTG binding factor

PBS- phosphate-buffered saline

PBST – PBS-triton

PDTC- poorly differentiated thyroid cancer

PFA- Paraformaldehyde

PI3K- phosphatidylinositol-4,5-bisphosphate 3-kinase

PKA- protein kinase-A

PKC- protein kinase C

PMA- phorbol 12-myristate 13-acetate

PPARγ- peroxisome proliferator-activated receptor γ

p-S6 – phosphorylated S6

PTC- Papillary thyroid carcinoma

PTTG- Pituitary tumor-transforming gene

PVDF- polyvinylidene difluoride

RAC1- Ras-related C3 botulinum toxin substrate 1

RAI- Radioiodine

Rap- Rapamycin

RAS- rat sarcoma

RET- rearranged during transfection

RNA- ribonucleic acid

RTK- receptor tyrosine kinase

RT-qPCR- quantitative reverse transcription PCR

SD- standard deviation

SDS- sodium dodecyl sulfate

SDS-PAGE- sodium dodecyl sulfate-polyacrylamide gel electrophoresis

sec- second

SLC5A5- solute carrier family 5, member 5

SMAD3- mothers against decapentaplegic homolog 3

T3- triiodothyronine

T4- thyroxine

TBE- Tris-borate EDTA

TBS- Tris-buffered saline

TBST- TBS-triton

TC- Thyroid cancer

TEMED- tetramethylethylenediamine

Tg- thyroglobulin

TGF-β- transforming growth factor beta

TLR-4- toll-like receptor 4

TNFα- Tumour necrosis factor α

TPO- Thyroid peroxidase

TRH- Thyrotropin releasing hormone

Tris-HCl- TRIS hydrochloride

TSH- thyroid stimulating hormone

TSHR- thyroid Stimulating Hormone Receptor

TTF-1- Transcription Termination Factor 1

TTF-2- Transcription Termination Factor 1

U- unit

UV- ultraviolet

V- Volt

w/ - with

w/o- without

ws- without stimulation

wt- wild-type

^{131}I- radioactive iodide

^{o}C- degree celsius

µ- micro

% (w/v)- concentration expressed in weight per volume

% (v/v)- concentration expressed in volume per volume

I. INTRODUCTION

1. Carcinogenesis

Over the past decades, a growing number of people have been diagnosed with cancer, which is considered one of the leading causes of morbidity and mortality worldwide (WHO *et al.*, 2017). Cancer research is yielding an increasing body of knowledge, reflecting an extensive effort currently being carried to treat this complex disease. Despite all the advances resulting from cancer research in its understanding, diagnosis and treatment, research must continue as it remains a major public health issue (Elsevier Research Inteligence, 2016).

Cancer is a multifaceted and complex disease which results from an abnormal and uncontrolled cell growth that ultimately may invade organs other than its primary site. It can develop in virtually any tissue or organ in the human and, consequently, there is more than a hundred types of cancer with different clinical causes and response to treatment (Cooper, 2000). Despite this heterogeneity, the study of oncological diseases has been focused on the common features between different types of cancer rather than in their differences.

Studies showed that this uncontrollable division of cells is a result of the gradual accumulation of gene mutations that have its roots on environmental and hereditary factors (Tomasetti *et al.*, 2017). Three classes of genes were identified as the main cancer-causing genes: the tumour supressing genes, associated with loss of function alterations; the oncogenes, characterized by a gain of function alterations and DNA repairing genes. Alterations in these genes lead to dysregulated cell processes that increase the probability to aquire additional mutations, which ultimately can turn healthy functioning cells into cancerous cells (Bishop *et al.*, 1996).

Research made on the biological mechanisms from which a normal cell grows into a tumorous cell involves a dynamic change in the genome that potentiates cancer development (Vogelstein *et al.*, 1993). Even though there is a complexity in the vast number of factors contributing to tumorigenesis, investigators Hanahan and Weinberg defined that almost all cancers comprise six common alterations in cell physiology: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan & Weinberg, 2000). To this date, four more hallmarks of cancer were added to the already existing ones: abnormal metabolic pathways, the immune system evasion, genome instability and inflammation (Figure I.1.) (Hanahan & Weinberg, 2011).

These traits enable cancer cells to ignore cell death signals, escape control and become invasive (Liotta *et al.*, 2001; Hanahan & Weinberg, 2011).

Figure I.1. The Hallmarks of cancer. This figure illustrates the six hallmark capabilities originally proposed along with two emerging hallmarks and two enabling characteristics. Adapted from Hanahan and Weinberg (2011).

2. Thyroid gland: structure, function and regulation

The thyroid gland stands as one of the largest endocrine glands, weighting between 15 to 20 grams in adults. It lies immediately below the larynx and surrounds the anterior and lateral aspects of the upper trachea. It has two lateral lobes united by the isthmus. Its size and appearance can vary depending on gender, hormonal status, functional activity and iodine uptake (Figure I.2.) (Hall *et al.*, 2011; Muro-Cacho *et al*, 2000).

The gland lays inside a fibrous capsule and is composed of spherical groupings of cells called follicles. These are the functional unit of the thyroid gland and are arranged around the colloids. The storage of thyroid hormones occurs mainly in these extracellular compartments, being one of the standout features of the thyroid gland that makes it unique, when comparing with other endocrine glands, since it has the ability to store larger quantities of hormones, instead of smaller quantities in the intracellular compartments (Muro-Cacho *et al.*, 2000; Ellis *et al.*, 2003).

There are two hormone producing types of cells. The parafollicular cells that are spread between the follicles and secrete calcitonin, an important hormone for calcium metabolism, also called C cells. The other group of cells are called follicular cells and these produce the major thyroid hormones, thyroxine and triiodothyronine, commonly called T4 and T3, respectively (Figure I.2.) (*Nilsson* et al., 2017). Both hormones increase the basal metabolic rate and have an impact on development of the vertebrates, as well as in the cardiovascular function (Mullur et al., 2014). The secretion of thyroid hormones is regulated by thyroid-stimulating hormone (TSH), secreted by the anterior pituitary gland which is controlled by thyrotropin releasing hormone (TRH) produced by the hypothalamus (Nussey *et al.*, 2001).

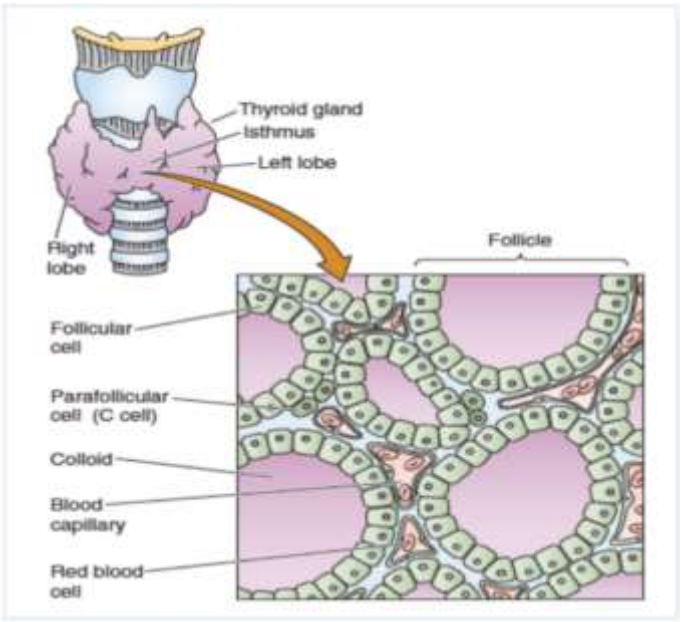

Figure I.2. Macroscopic and microscopic structure of the thyroid gland. The gland sits in the lower anterior neck around the front of the trachea. A single layer of follicle cells form the thyroid epithelia, and are filled with colloid. In the space between the follicles there can be found blood vessels and C cells grouped together. Adapted from Boron *et al*. (2012).

Thyroid hormone synthesis depends on iodine, since both T3 and T4 have iodine in their chemical structures. The synthesis of T3 and T4 starts when the iodide ions (I^-) are carried through bloodstream and then are transported into the thyroid follicles through the sodium iodide symporter (NIS) (Rousset *et al.*, 2015). In the lumen, the oxidation of iodine occurs mediated by thyroperoxidase (TPO). Iodine is added to the tyrosine residues of thyroglobulin (Tg) that is secreted by exocytosis from the follicular cell's cytoplasm to the lumen upon stimulation with TSH, for the production of T3 and T4. Both hormones can then be stored in large quantities inside the follicles, as components of Tg or, when needed, Tg is endocyted into the follicular cells where the lysosomes merge with endocytic vesicles. Inside these structures hydrolysis by proteolytic enzymes occur, leading to the release of T3 and T4 from Tg into the interstitial space and then, into the bloodstream (Figure I.3.) (Mansourian *et al.*, 2011).

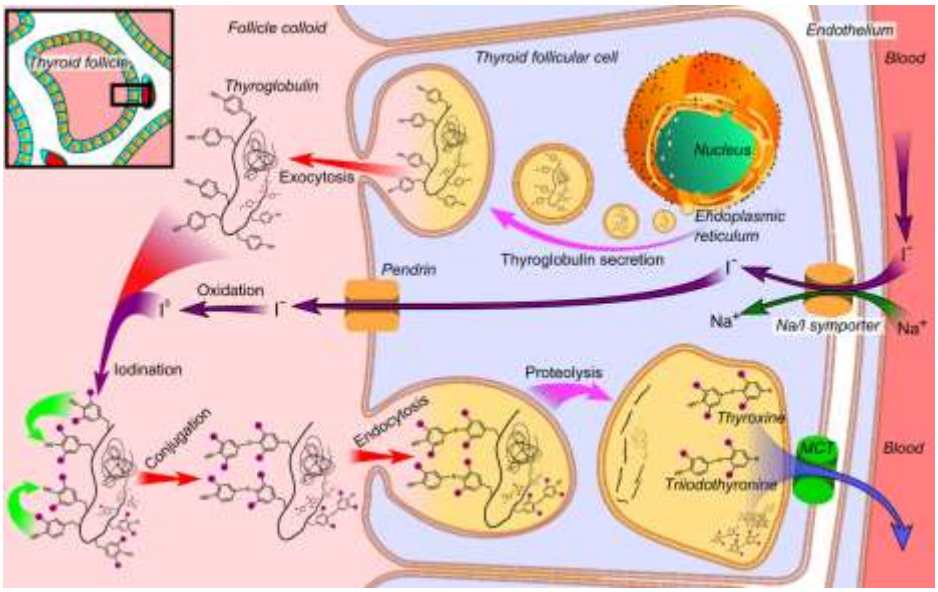

Figure I.3. Thyroid hormone synthesis TSH stimulates the synthesis of thyroid hormones. T4 and T3 synthesis starts when I- is uptaken into the follicular cells by NIS. In the colloid, I^- is oxidized to I0 by TPO, which then attaches to tyrosine residues in Tg. The iodinated tyrosines are linked together to form T4 and T3 and ready to be transported from the colloid to the follicular cells where Tg is hydrolysed for both hormones to be released into the blood circulation. From Häggström, 2014.

2.1. Thyroid cancer

Thyroid cancer (TC) incidence has been rising worldwide over the past years.It is higher in women and represents the most common endocrine malignancy, accounting for 90% of all endocrine malignancies (Lim *et al*., 2017; Siegel *et al*, 2013). Particularly in Portugal, there has been a significant annual increase in its occurrence of 2 to 9 per cent between 1988 and 2011, having the highest incidence amongst the other European countries or even the world (Raposo *et al.*, 2016). The reason for this increase remains unclear and cannot be justified only by overdiagnosis due to more sensitive methods. The rise in TC incidence is most likely due to a combination of factors and may be a consequence of increased population exposure to radiation and to other unidentified carcinogens (DeLellis *et al.*, 2004; Pellegriti *et al.*, 2013).

When considering thyroid malignancies, there is a wide range of clinical behaviours, from indolent to highly life-threatening cancers (Kebebew *et al*., 2000). Most are differentiated follicular cell-derived thyroid carcinomas (DTC) and comprise two main subtypes, which depend on the histological features: the papillary thyroid carcinoma (PTC) and the follicular thyroid carcinoma (FTC) (Kebebew *et al*., 2000). PTC is the most frequent thyroid cancer, comprising 80-85% of all cases (Nifikorov *et al*. 2011). Even though most of the time its prognostic is favourable, disease recurrence occurs in about 40% of the patients (Kebebew *et al*. 2000). FTC represents 10-15% of all thyroid cancers (Nifikorov *et al*. 2011). Another subtype of TC is the medullary thyroid carcinoma (MTC) which arises from thyroid parafollicular cells. Albeit it accounts for only 3-5% of all thyroid carcinomas, it is associated with up to a 13.5% mortality rate (Kuo *et al*., 2017). Fewer cases of thyroid carcinomas have a poorly differentiated (PDTC) or even undifferentiated or anaplastic (ATC) phenotype (DeLellis *et al*., 2004), which is the most aggressive form of TC.

The standard of care for thyroid carcinoma is surgery, despite the cells' histological features (Sonkar *et al*., 2010). Regarding DTCs, these usually have a good response to the standard therapeutic protocol which includes thyroidectomy, TSH suppressive therapy and, in selected patients, ablative radioactive iodine (RAI) treatment (Sonkar *et al*., 2010; Soares *et al*., 2014). In

fact, the use of RAI is the initial systemic treatment of choice for TC metastatic disease. However, about 10% of patients with advanced forms of thyroid carcinoma are refractory to radioiodine, due to de-differentiation of thyroid cancer cells which will affect NIS regulation, decrease its functional expression and reduce iodide uptake (Sonkar *et al*., 2010; Spitzweg *et al.*, 2014). Since there are no alternative effective therapies available, the 10 years survival rate decreases from approximately 90% to 10% and the clinical management of these patients becomes challenging (Ganly *et al*., 2015; Schmidt *et al*., 2017). In this project we aimed to find strategies to enhance iodide uptake efficiency in order to allow the RAI therapy to be applied to RAI-refractory thyroid cancers, using a non-transformed and a DTC cell line as models.

2.1.1. Papillary Thyroid Carcinoma

PTC is the most prevalent type of DTC (Nifikorov *et al*. 2011). Its incidence is higher in women and is most prevalent in older people, but it can also manifest in younger patients between 20-50 years old. PTCs may also occur in children and radiation exposure increases the risk of developing this cancer subtype (DeLellis *et al*., 2004).

Somatic genetic alterations of PTC have been largely studied throughout the years with the purpose of finding the disease driver mutations. It resulted in the identification of recurrent mutations in known oncogenes, such as BRAF and RAS (Nifikorov *et al.*, 2011, Saji *et al.*, 2010). RET/PTC rearrangements collectively describe a series of chimeric-transforming oncogenes generated by fusion of the RET proto-oncogene with the 5′ terminal sequence of one of different heterologous genes. These are commonly found in childhood PTC regardless of radiation history and in adult patients with a history of radiation exposure (Hamatani *et al*., 2008). Protein products of these mutated/rearranged genes are commonly part of the mitogen-activated kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) / protein kinase B (AKT) / mammalian target of rapamycin (mTOR) signalling pathways (Giordano *et al.*, 2018).

2.1.2. Follicular Thyroid Carcinoma

FTC has been described as a malignant epithelial tumour with evidence of follicular cell differentiation (Bhaijee *et al.*, 2011; DeLellis *et al.*,2004).

Some of the factors influencing FTC's prognosis include the age of the patient, staging, tumour size, completeness of surgery and efficacy of radioiodine treatment (Sobrinho-Simões *et al.*, 2011). Another important aspect when considering the prognosis is the degree of invasiveness of the tumour, ranging from minimally invasive to widely invasive tumours, with the prognosis worsening in that order (Muro-Cacho *et al.*, 2000). The most common mutations found in this category of tumours are aneuploidies, RAS mutations and PAX8-PPARγ rearrangements (Sobrinho-Simões *et al.*, 2005).

3. NIS: molecular and functional characterization

For over a century it has been known that thyroid follicular cells are able to accumulate iodide since it was reported that the thyroid gland could concentrate I^- with a factor of 20 to 40 in relation to the concentration found in the plasma under physiological conditions. Consequently, it was predicted to exist a I^- molecular transporter in these cells, which was identified and characterized in 1996, first in rat and then in human. It was named sodium-iodide symporter (NIS or SLC5A5, solute carrier family 5, member 5) and, since its identification, many advances have been made with the effort to unravel its structure and function (Carrasco *et al.*, 1993; Dai *et al.*, 1996).

NIS is a glycoprotein integrated in the basolateral plasma membrane of the thyroid follicular cells. It is also present in other tissues such as the salivary glands, gastric mucosa, kidney and the lactating mammary gland, but in much lower levels than those found in thyroid (Carrasco *et al.*, 1993; Kogai *et al.*, 2012). It has 618 aminoacids in rat and mouse and 643 aminoacids in human and pig, both amino acid sequences sharing 84% identity and 92% similarity. NIS has 13 putative transmembrane domains, an extracellular amino-terminus and an intracellular carboxyl-terminus (Figure I.4.) (Dóhan *et al.*, 2003; Riesco-Eizaguirre *et al.*, 2006). The final protein has three potential N-linked glycosylations culminating in a 70-90 kDa molecular weight. However, the lack of N-linked glycosylation does not impair its function, stability or retention in the plasma membrane (Levy et al., 1998).

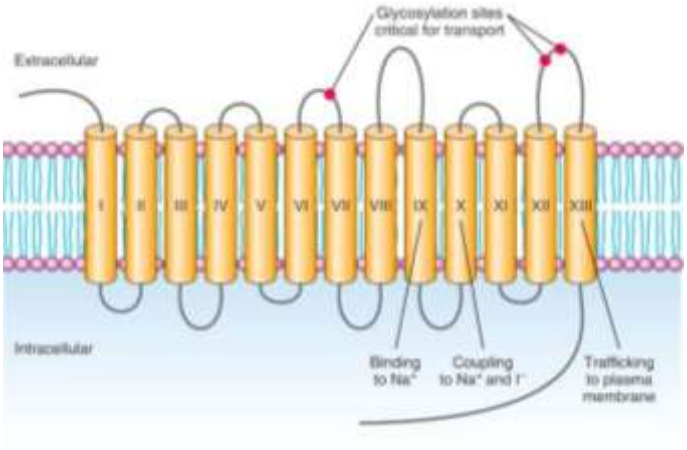

Figure I.4. Secondary structure of the human NIS protein. The illustration displays the predicted secondary structure of NIS. This model shows 13 transmembrane domains labelled by a roman numeral with N terminus located extracellularly and C terminus located intracellularly. Transmembrane segments are represented by cylinders. Adapted from Spitzweg *et al*., 2010.

Through experiments made by Levy *et al*. (1997) in the thyroid-derived rat FRTL-5 cells, they found that NIS is first synthesized as a precursor and then is core glycosylated in the endoplasmatic reticulum, thus evolving into a mature protein. After that, NIS reaches the plasma membrane where it starts playing its function by uptaking I^-.

When stimulated by TSH, NIS catalyzes the active transport of one I^- together with two Na^+ ions from the instersticium into the cell against an electrochemical gradient. On the other hand, the efflux of I^- to the follicular lumen is attained through passive transport (Eskandari *et al.*, 1997). The active inward transport of I^- is achieved through the energy generated by a Na^+/K^+ATPase which drives Na^+ on the opposite direction (Dohán *et al.*, 2003). The process of concentrating I^- for extended periods of time is unique to the thyroid gland, due to the process of T3 and T4 incorporation, as previously mentioned in section 2 “Thyroid gland: structure, function and regulation” (Carrasco *et al.*, 1993).

The number of *in vitro* and *in vivo* studies involving the regulation of NIS expression has increased in recent years revealing that NIS has a complex transcriptional and post-transcriptional regulation (Alotaibi *et al*., 2017).

3.1. Transcriptional regulation of NIS

Gene transcription is specific to each cell type and depends on a certain combination of transcription factors. For thyroid cells, in particular, there are three transcription factors described

as central: TTF-1, TTF-2 and PAX8, which are involved in the transcriptional control of several participants of thyroid hormone syntesis including Tg, TPO and NIS (Zhang *et al.*, 2006).

NIS expression and functional regulation is made by different molecules and pathways at both transcriptional and post-transcriptional levels. The main hormonal regulator is TSH that acts via a cyclic adenosine monophosphate (cAMP) pathway resulting in the activation of NIS upstream enhancer (NUE) (Kogai *et al*. 2012).

3.1.1. Regulation of NIS by TSH

TSH is the main hormonal regulator of thyroid function and stimulates iodide uptake in the thyroid. The actions of TSH are mediated by the cAMP pathway. Its activation starts by TSH binding to its receptor (that is connected to G proteins), which will activate the adenylate cyclase enzyme leading to the raise of intracellular levels of cAMP. cAMP stimulates the NUE through both protein kinase-A (PKA)-dependent and independent pathways. PKA can phosphorylate the cAMP-responsive element binding protein (CREB) which then binds to the cAMP responsive element (CRE) leading to the activation of NUE (Kogai *et al*. 2012).

PAX8, on the other hand, regulates the transcription of thyroid specific genes such as TSHR, Tg, TPO and NIS. PAX8 binding to NUE, occurs via stimulation with TSH, which leads to the reduction of PAX8 through redox effector factor-1 (Ref-1), resulting in the PAX8 binding to its *cis*-elements (Kogai *et al*. 2012).

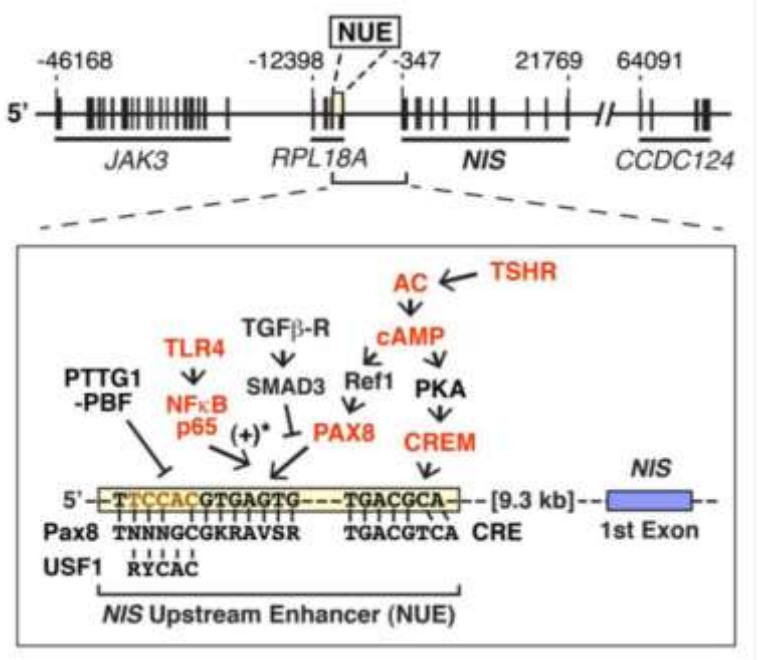

Figure I.5. Regulation of NUE Schematic figure of the human chromosome 19p around the NIS gene locus and the TSHR signalling pathways to NUE which mainly regulate NIS expression in thyroid cells. From Kogai *et al*., 2012.

It was observed, in human and in rat thyroid cells, that TSH up-regulated NIS expression and iodide uptake through the cAMP-mediated pathway. TSH withdrawal, on the other hand, produced a reduction of intracellular cAMP levels and, consequently, of iodide uptake in FRTL-5 cells (Marcocci et al. 1984; Kogai et al. 1997; Ohno et al. 1999).

3.1.2. Regulation of NIS by other signal transduction pathways

The TSH-TSHR-cAMP-PKA pathway has been recognized as the central and most important signalling pathway behind thyroid cells development. However, there are other signalling pathways implicated in NIS expression, namely the MAPK and PI3K signalling pathways

MAPK and PI3K pathways were found to be overactivated in many thyroid cancers (Knauf & Fagin 2009, Xing 2010). Moreover, as a consequence of this activation, NIS expression is downregulated in several thyroid cancer derived cell lines (Cass & Meinkoth, 2000, Knauf *et al*. 2003, Zaballos *et al*. 2008).

Mutations that activate the MAPK pathway (Figure I.6), are associated with approximately 70% of papillary carcinomas (Nifikorov, 2008). MAPK pathway can be activated by external stimuli, such as growth factors and mitogens that bind to their respective plasma membrane receptors, such as receptor tyrosine kinase (RTK) (Shaw *et al*., 2006). This step allows the small GTPase Ras to exchange its GDP-associated molecule for a GTP, switching to the active state. Active Ras activates MAP3K that activates MAP2K, which in turn activates MAPK. From here, MAPK can activate a set of transcription factors associated with proliferation, differentiation, motility and apoptosis (Katz *et al*., 2007; Downward, 2003). Mutations in BRAF, RAS and RET/PTC are responsible for activating the MAPK signalling pathway in cancer cells. By being constitutively activated it will consequently lead to an uncontrolled cell division and resistance to apoptosis, resulting in cancer progression (De Luca *et al*., 2012). The BRAF mutation leads to the downregulation of NIS through the MEK-ERK pathway and through the transforming growth factor (TGF)-β/SMAD3 pathway (Riesco-Eizaguirre *et al*., 2009). Both are responsible for the negative effect on PAX8 / NUE activation, which will result in downregulation of NIS expression. Some approaches performed *in vitro* to improve iodide uptake included the inhibition of BRAF through small interfering RNAs under stimulation of TSH and the treatment with selumetinib, an inhibitor of the mitogen-activated protein kinase kinase (MEK1/2) that acts downstream of BRAF (Kleiman *et al*., 2013). Consistently, in a recent trial with selumetinib, a meaningful increase in RAI uptake was observed in patients with RAI-refractory TC carrying MAPK activating mutations, illustrating the clinical relevance of targeting this pathway to enhance the efficacy of RAI therapy (Sherman *et al*., 2013).

The PI3K/Akt pathway (Figure I.6.) is involved in normal cell physiology, being responsible for processes associated with cell cycle regulation. Mutations leading to aberrant activation of the pathway have been described to play an important role in thyroid tumorigenesis. In fact, there are studies reporting an association between this pathway and thyroid cancer, especially in FTC and ATC (Xing *et al*. 2010). PI3K are lipid kinases that phosphorylate the 3-hydroxyl group of phosphoinositides and, similar to the MAPK pathway, are activated by the binding of growth factors and chemokines to their RTKs (Carpenter *et al*., 1993). Ultimately its activation results in the activation of Akt and subsequently on the activation of the mTOR complex. This protein complex exists in two forms, mTORC1 and mTORC2. Mutations involving this pathway contribute to carcinogenesis, together with BRAF mutations. The alterations seen in the pathway effectors were reported to increase tumour's aggressiveness (Xing *et al*. 2010). Studies suggested that an amplification of a gene coding for a catalytic subunit of PI3K, especially present in DTC, could trigger the activation of PI3K/mTOR signalling pathway in thyroid cancer (Wu *et al*., 2005). Moreover, it was found that levels of phosphorylated Akt were increased in FTC (Ringel *et al*., 2001). In TSH stimulated thyroid cells, the activation of this pathway reduces NIS transcript levels affecting the iodide uptake by these cells. Inhibitors for PI3K were shown to increase NIS expression. Accordingly, these drugs could be used to enhance RAI uptake in TSH-stimulated thyrocytes (Kogai et al., 2008; Liu et al., 2012).

Nuclear factor-κB (NF-κB), was reported to also participate in thyroid physiology as an important molecule for the expression of the main thyroid-specific genes, such as PAX8, Tg, TTF-1 and TPO, but also for the expression of NIS (Giuliani *et al*., 2018). Nevertheless, the role

of NF-κB pathway in both normal and neoplasic thyroid contexts is still poorly clarified and its role on NIS expression remains to be deciphered. This subject will be further discussed below in section 4.2.

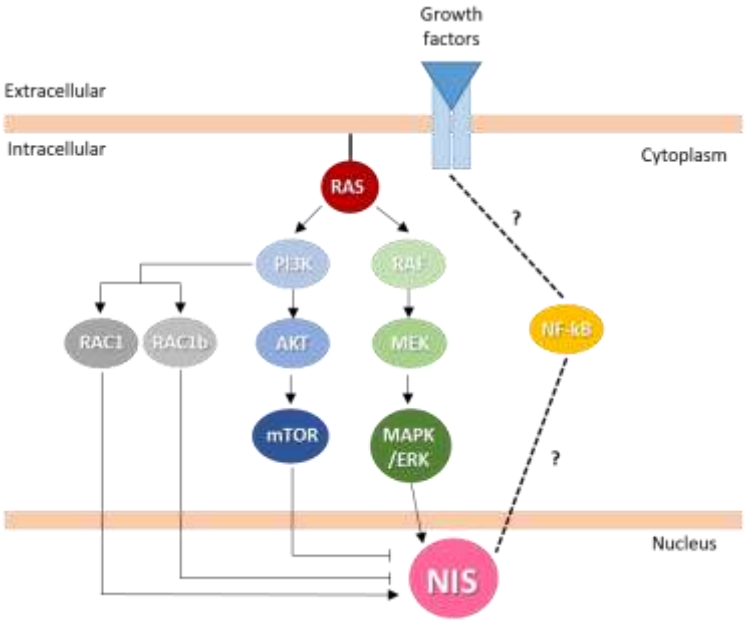

Figure I.6. Signal transduction pathways in NIS regulation Schematic representation of the signal transduction pathways involved in the regulatory mechanisms of NIS expression which include the MAPK pathway and the PI3K/Akt/mTOR pathway that require RAS activation. Also, the NF-κB pathway whose role on NIS expression is still to be deciphered.

3.2. NIS protein expression

In order to be fully functional, NIS must be properly located at the plasma membrane. This is relevant for iodide uptake and consequently for radioiodine therapy in thyroid cancer. Studies have reported that in several thyroid tumors, even when NIS mRNA expression is high, the correspondent protein expression is low at the cell surface. Thus, the decreased iodide uptake has been also assigned to post-translational regulatory mechanisms, such as the impairment of NIS translocation or retention at the plasma membrane (Kogai *et al*., 2012).

TSH and iodide were shown to affect NIS translocation to the plasma membrane (being this promoted by TSH and inhibited by excess of iodide). Studies have shown that when FRTL-5 cells were stimulated with TSH, NIS was spatially distributed across the plasma membrane. However, when TSH stimulation was halted, NIS was mostly found in intracellular compartments (Riedel *et al*., 2001). These findings support a role for TSH in the posttranslational regulation of NIS.

Another factor implicated in NIS posttranslational regulation is the PTTG binding factor (PBF), a NUE regulator. In fact, the expression of PBF was found to be particularlly high in thyroid cancer and was associated with low expression of NIS at the plasma membrane. In addition, PBF was found to interact and co-localize with NIS, modulating NIS intracellular traffic by impairing its membrane translocation (Stratford *et al*., 2005; Smith *et al*., 2009).

In conclusion, functional NIS expression can be enhanced transcriptional and/or post-translationally. The study of signalling pathways contributing to NIS functional up-regulation

may lead to the development of strategies to allow the application of RAI therapy to RAI-refractory thyroid cancer.

4. NF- κB: key-players, regulation and function

As mentioned above, even though the NF-κB signalling pathway has been implicated in the expression of several thyroid-specific genes, its role on NIS expression remains elusive.

NF-κB is a family of transcription factors, made up of heterodimers or homodimers that play an important role in many cellular processes including the regulation of cell proliferation and survival (Nicola *et al.*, 2010). In mammalian cells there are five studied members of the NF-κB family: p65 (RelA), RelB, c-Rel, p50 and p52. All these share an N-terminal Rel homology domain (RHD) which is responsible for dimerization, DNA binding and nuclear translocation, having different specificities in their different combinations (Ghosh *et al.*, 2002).

In its inactive form, the NF-κB proteins are sequestrated in the cytoplasm by a family of inhibitory proteins called inhibitor of kappa B (IκB) that maintain them inactive by masking their nuclear localization signal (NLS). However, when exposed to stimuli such as lipopolysaccharide (LPS), interleukin-1 (IL-1), TNF-α or phorbol esters, the degradation of IκB will take place exposing the NLS of the NF-κB complex which is then able to migrate to the nucleus and activate its target genes (Hacker *et al.*, 2006. Pahl *et al.*, 1999). One of these target genes is NF-κB's own repressor, IκB. This will generate a negative feedback loop, as the IκB will once again inhibit the activity of NF-κB. Hence, the NF-κB activation is mainly cyclical when continuously induced (Di *et al.*, 2012).

There are two general pathways through which NF-κB is activated, the canonical and the non-canonical, also called the classical and the alternative pathways, respectively (Gilmore *et al.*, 2006).

The canonical NF-κB pathway (Figure I.6.) involves NF-κB dimers such as p50/RelA which are kept in the cytoplasm by interacting with IκB-α. It is frequently activated by inducers such as IL-1b and TNF-α cytokines, but also inducers such as LPS and phorbol esters are able to activate the canonical NF-κB pathway. The binding of these molecules to the respective receptors leads to an activation of IκB kinase subunit beta (IKKβ) in the IKK complex which can then phosphorylate IκB-α on the serine residues S32 and S36. IκB-α will then go through polyubiquitination and subsequently proteasomal degradation, exposing a nuclear localization signal (NLS) in the NF-κB subunit that will allow its translocation to the nucleus and activation of the target gene transcription (Gilmore *et al.*, 2006; Carrá *et al.*, 2016).

The activators of the non-canonical pathway (Figure I.6.) include non-death receptor members of the TNF receptor family such as CD40, B-cell-activating factor (BAF), lymphotoxin beta (LTb), and some viral proteins such as LMP-1 from the Epstein-Barr virus (EBV). After the binding of one of these molecules to the respective receptor, the NF-κB-inducing kinase (NIK) is activated which in turn phosphorylates the IKKα complex activating it. IKKα will phosphorylate two serine residues adjacent to the repeat C-terminal IκB domain of p100 subunit. Consequently, p100 suffers a partial proteolysis and releases the p52/RelB subunit, which is then free to regulate the transcription of its target genes (Gilmore *et al.*, 2006; Carrá *et al.*, 2016).

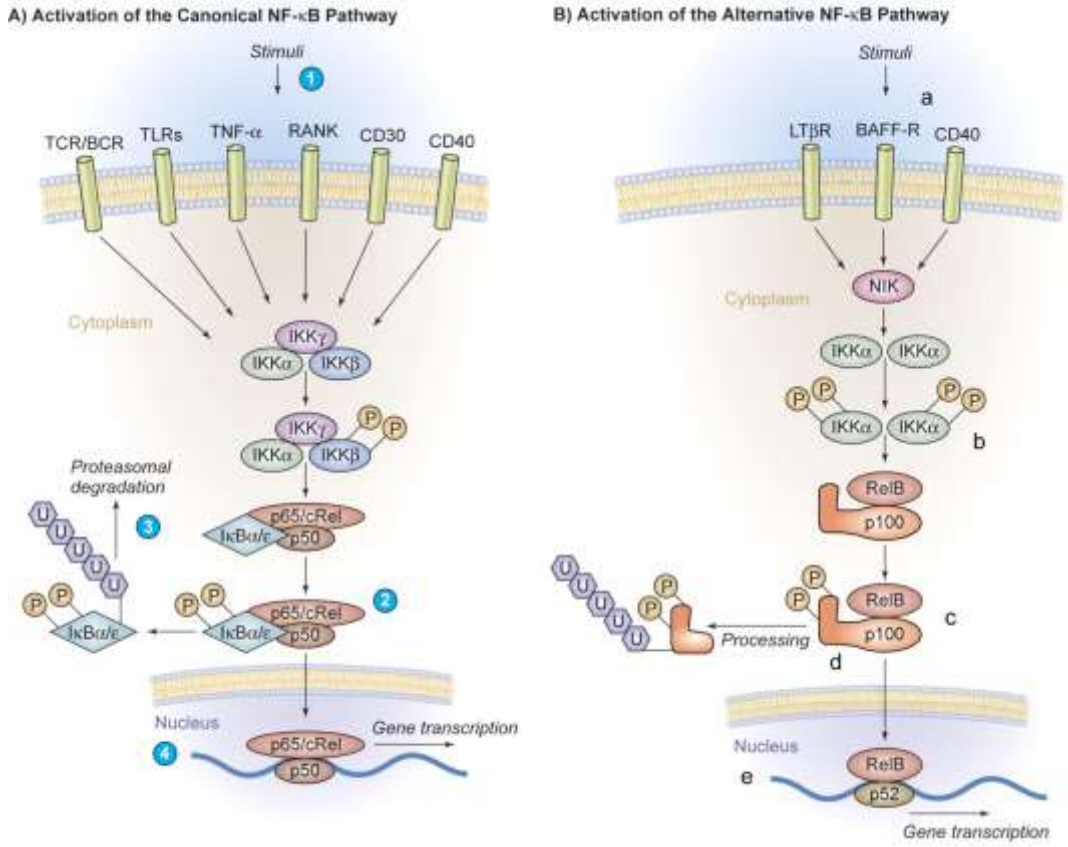

Figure I.7. Canonical and alternative pathway of NF-κB activation. In the canonical pathway NF-κB is retained in the cytoplasm bound to its repressor IκB until stimuli activate IKK that in turn phosphorylates IκB, inducing its proteolytic degradation, culminating in the activation of the NF-κB dimer p50/RelA. The non-canonical pathway encompasses the activation of IKKα that in turn activates the IκB domain of p100 resulting in the release of the p52/ReLB dimer. From Jost P. *et al*. 2007.

4.1. NF-κB role in thyroid cancer

Studies have demonstrated a role of the transcription factor NF-κB in cancer inflammatory microenvironment, by its ability to suppress apoptosis and to regulate cell-cycle progression. Inflammation-associated cytokines affect the pathways that modulate NF-κB function and activity and, subsequently, there will be great changes in cell-specific gene expression (Vlahopoulos *et al*., 2017). NF-κB is responsible for regulating the expression of cytokines and adhesion factors that control interactions between neighbouring cells and its communication with the immune

system. So, by activating this transcription factor, alterations in tissue function and composition will take place as well as alterations in the activity of specific metabolic and pro-tumorigenic pathways (Vendramimi *et al.* 2017). In addition, one of the consequences of the disruption of NF-κB control is a more intense and prolonged cancer-associated inflammatory gene expression in the tumour microenvironment, allowing these cells to escape immune surveillance and to metastize (Spiros *et al*., 2017).

NF-κB has been recently shown to play an important part in thyroid cancer. Some oncogenic proteins involved in the thyroid carcinogenesis process include RET, RET/PTC, RAS and BRAF, that were shown to be able to induce NF-κB activation in follicular, papillary and medullary thyroid carcinomas. In addition, a constitutive deregulated NF-κB activity was shown to occur in anaplastic thyroid carcinomas (Pacifico et al., 2010).

4.2. NF-κB and NIS expression

As mentioned above, NF-κB participates in thyroid physiology as an important regulator of the expression of main thyroid-specific genes (Giuliani *et al*., 2018). Recent studies addressed whether the activity of this transcription factor has an impact on NIS expression. In fact, it was found that the Gram-negative bacterial endotoxin, LPS, which binds to TLR-4 receptor, greatly increased NIS mRNA expression as well as iodide uptake under stimulation of TSH in FRTL-5 cells. This occurred through the activation of the NF-κB, particularly of the p65 NF-κB subunit, which physically interacts with PAX8 that, in turn, activates NUE, the promoter of NIS transcription. This suggests a role of the canonical NF-κB pathway on NIS expression activation (Giuliani *et al.*, 2018; Kogai *et al*., 2010; Nicola *et al.*, 2009; Nicola *et al*., 2010). On the other hand, TNF-α is widely recognized as a potent activator of the canonical NF-κB pathway (Li *et al*., 2014). Yet, this cytokine has been shown to downregulate up to 70% NIS mRNA expression in normal rat thyroid cells (Spitzweg *et al.*, 1999). This raises the question of whether NF-κB activation plays a role in the impact of TNF-α on the negative regulation of NIS.

In addition, previous studies from the host lab showed an association between RAC1b and the NF-κB pathway and between RAC1b and NIS. RAC1b is a hyperactive variant of the small GTPase RAC1, a member of the RAS superfamily of small GTP-binding proteins. It was shown that RAC1b has an important role in PTC development contributing to apoptosis resistance through NF-κB activation (Faria *et al*., 2017b). Moreover, it was also shown that in PCCL3 cells, ectopic overexpression of RAC1b was inversely correlated with NIS mRNA levels, suggesting that RAC1b played a role in inhibiting NIS expression (unpublished work). These studies led us to hypothesize that NF-κB could play a role on regulating NIS expression. In this project, we aimed to further clarify the effect the canonical NF-κB pathway may have on NIS expression.

5. Aim of the thesis

This project aims to characterize the pathways leading to the enhancement of NIS expression. This approach could ultimately contribute to increase the radioiodine uptake efficiency with prospects of maximizing radioiodine therapy efficacy in RAI-refractory thyroid carcinomas.

Having this goal in mind, our project focused on two main objectives:

1. To investigate the role of the NF-κB canonical pathway on the regulation of NIS mRNA expression, by determining the effect of TNF-α on NIS regulatory mechanism and wether this relies on the activation of the canonical NF-κB pathway.

2. To assess the effect on NIS expression of simultaneously targeting PI3K and mTOR in both non transformed thyroid PCCL3 cells as well as in K1 cells, representative of a thyroid cancer cell system with mutated PIK3CA gene (resulting in constitutive activation of PI3K signalling). With this purpose, the inhibitors LY294002 (PI3K inhibitor), Rapamycin (mTORC1 inhibitor) and BEZ-235 (PI3K/mTOR dual inhibitor) were used.

II. MATERIALS AND METHODS

1. Cell Culture

For this study we used two commercially available cell lines were used, PCCL3 (RRID:CVCL_6712) which is a continuous line of Fischer rat's thyroid follicular cells, and K1 (RRID:CVCL_2537) which is a human papillary thyroid carcinoma derived cell line.

Cells were maintained at 37°C in a 5% humidified CO_2 environment and, when they reached the optimal confluence (80% to 100%), were detached with 0.05% trypsin/EDTA (Gibco) and replated in new flasks at 1:3 to 1:5 dilution. PCCL3 cells grew in Coon's F-12 modified liquid medium (*EuroClone*) (maintenance medium) supplemented with 5% fetal bovine serum (FBS) (*Gibco*), 10 μg/mL of insulin (*SIGMA*), 5μg/mL of Apo-Transferrin (Apo-T) (*SIGMA*) and 0.1 UmL of TSH (*SIGMA*). K1 cells grew in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM: F12, 1:1) (*Gibco*) with 0.365 g/L of L-Glutamine and suplemented with 10% (v/v) FBS (*Gibco*), 1% (v/v) GlutaMax (*Gibco*), 1.25% (v/v) sodium bicarbonate (*Gibco*) and 0.5% (v/v) of sodium pyruvate (*Gibco*).

To perform the drug treatments in the PCCL3 cell line, cells were seeded at 60% confluence in 12 well plates (*Corning*) and subjected to serum-starvation medium (F12 Coon's Modification medium supplemented with 0.2% (v/v) FBS and 2 μg/mL of Apo-T) in the absence of TSH for 48h which allowed cells to be quiescent before the experiments. Starved cells were then subjected to TSH stimulation for 24 hours (1 U/mL of TSH in serum-starvation medium) and next treated with 10 ng/mL TNF-α (*SIGMA*), 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (*SIGMA*) for different periods of time (30-60 minutes to 24 hours). When applicable, cells were also treated with 10 μM BMS-345541 (*SIGMA*) for 6 hours**.**

To test the different pathway inhibitors, starvation-medium was changed to stimulation-medium (F12 Coon's Modification, 5% FBS, 5μg/mL of Apo-T and 1 U/mL of TSH) and PCCL3 and K1 cells were treated with 100 nM BEZ-235 (*SIGMA*) for 6 hours, 50 μM LY294002 (*SIGMA*) for 6 hours, or 100 nM Rapamycin (*SIGMA*) for 6 hours.

2. RNA extraction

RNA extraction was performed using the ready-to-use reagent TripleXtractor (*GRiSP Research Solutions*) according to the manufacturer's instructions. Throughout this protocol an Eppendorf centrifuge 5417C (rotor radius = 95 mm) was used and the RNA extracted was quantified resorting to a NanoDrop 2000 spectrophotometer (*Thermo Scientific*) and could then be immediately used or stored at -70°C.

3. cDNA synthesis

cDNA (complementary DNA) was synthetized from 2 μg of RNA by reverse transcription carried out by reverse transcriptase RevertAid Reverse Transcriptase (*Thermo Scientific*). Firstly, it was added 0.1 μL of random primers (3 μg/μL) (*Roche*), 0.8 μL of deoxynucleotides (dNTPs, 25 μM) (*GE Healthcare*) and bidestilled water (ddH2O) making a total volume of 15 μL. This mix was incubated at 65°C for 10 minutes, allowing the denaturation of the secondary structures of RNA that may exist and be an obstacle to cDNA synthesis. Then, a reaction mixture containing 4 μL of Reverse Transcriptase Buffer 5x (*Thermo Scientific*), 0.5 μL of Revert Aid Transcriptase 200 U/μL (*Thermo Scientific*) and 0.5 μL of RNAse OUT (Recombinant Ribonuclease Inhibitor, 40U/μL) (*Invitrogen*), was added to each sample and then incubated at 25°C for 10 minutes, 42°C

for 60 minutes and 70°C for another 10 minutes. All the incubation steps were performed by a 2720 Thermal Cycler (*Applied Byosystems*).

4. Polymerase Chain Reaction

In order to analyse the expression of IκB-α and NIS, PCRs were used starting from the cDNA previously synthetized and using a set of primers specific to the amplification of these genes. For NIS, the following primers were used: NIS RAT F (F-forward) (5'-TCCTCACAGGCCGTATCTCA) and NIS RAT R (R-reverse) (5'-GAAGGAACCCTGGAGGCAC), generating a PCR product of 314 bp; NIS human F (5'-CCCCAGACCAGTACATGCC) and NIS human R (5'-GGCCGATCCGTAGATGAGTG) which generated a PCR product of 299 bp. As for the IκB-α gene, the primers used were: IκB-α RAT F (5'-CTCCATCTTGCCTGTGAGCA) and IκB-α RAT R (5'-CCCCACACTTCAACAGGAGC) which generated a PCR product of 280 bp.

The PCR reaction mix consisted of 12.5 μL of PCR buffer 1x (Table VII.1), 0.25 μL of the reverse and forward primer (10 μM) and 0.1 μL of Taq Polymerase to which 1 μL of cDNA was added. The amplification was performed in a 2720 Thermal Cycler following the thermocycling conditions as described in Table VII.2.

To evaluate the expression of NIS and IκB-α the amplified PCR products were resolved by electrophoresis on a 2% (w/v) agarose gel in TBE (Tris-borate-EDTA) buffer 1x (diluted from TBE buffer 10x, *National Diagnostics*). PCR products were stained with 0.5% (v/v) ethidium bromide (*Invitrogen*) and visualized through exposure to UV light using a transiluminator GenoSmart (*VWR*).

5. Quantitative (real-time) - PCR (qRT-PCR)

NIS and IκB-α expression levels were quantified by SYBR Green based qRT-PCR, using Xpert Fast SYBR (*Grisp*) and following manufacturer's instructions.

These amplification reactions were performed using the Light Cycler 480II (Roche), following the conditions described in Table VII.3. and were carried out in triplicate for each sample. HPRT1 and TBP were used as endogenous control genes for rat and human, respectively.

The reaction mixture of 10 μL final volume contained 5 μL of SYBR Green, 3.5 μL of ddH_2O, 0.25 μL of each primer and 1 μL of cDNA. The primers used in order to amplify NIS, IκB-α, HPRT1 and TBP were the following: NIS RAT F (5'-TCCTCACAGGCCGTATCTCA) and NIS RAT R (5'–GAAGGAACCCTGGAGGACAC); NIS human F (5'-CCTGCTAACGACTCCAGCA) and NIS human R (5'-CCAGGGCACCGTAATAGAGA); IκB-α RAT F (5'-TCCTCACAGGCCGTATCTCA) and IκB-α RAT R (GACACGTGTGGCCGTTGTAG); HPRT1 F (5'-GCTGAAGATTTGGAAAAGGTG) and HPRT1 R (5'-AATCCAGCAGGTCAGCAAAG) and TBP F (5'-TGCACAGGAGCCAAGAGTGAA) and TBP R (5'-CACATCACAGCTCCCCACCA).

6. Protein extraction, SDS-PAGE eletrophoresis and Western Blot

Protein extraction of PCCL3 and K1 cells was carried out from 12 well plates by adding 50 μL of sample buffer (Table VII.1.) to each well. Samples were heated to 95°C for 10 minutes, with the aim of denaturate the proteins and after they were resolved on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Table VII.1.), for approximately 1 hour at 0.02 A. Next, the proteins were transferred, using a Mini Trans-Blot Electrophoretic Transfer

Cell (*Bio-Rad*), at 100 V for 1 hour to a PVDF membrane (polyvinylidene difluoride) (*Bio-Rad*), activated with methanol. The primary antibody used was anti-phospho-S6 (rabbit) (*Thermo Scientific*), at a 1:2000 dilution in TBST and anti-β-actin (mouse) (*Thermo Scientific*), diluted at 1:10000 in TBST milk, both incubated at 4°C, overnight with agitation. The next day the membrane was incubated with the secondary antibody anti-rabbit (*Thermo Scientific*) and with the secondary antibody anti-mouse (*Thermo Scientific*), both diluted at 1:5000 in TBST milk (Table VII.1.) for 1 hour at room temperature with agitation. The proteins were detected through chemiluminescence of the ECL substrate (Table VII.1.) followed by exposure and image acquisition on a ChemiDoc XRS System (BIO-RAD).

7. Confocal immunofluorescence microscopy

Coverslips were placed on 12 well plates and PCCL3 cells were let grown on them in the same conditions described in the "Cell Culture" section. The coverslips were washed 2 times in PBS 1x (*Gibco*), fixed with paraformaldehyde (PFA) at 4% (v/v) for 30 minutes at room temperature. Next, the cells were permeabilized with 0.5% Triton X-100 PBS 1x for 15 minutes and then washed with 0.05% Triton X-100 PBS 1x, 3 times, 5 minutes, with agitation. Following that, cells were placed in methanol for 20 minutes at -20ºC and then were washed with 0.05% Triton X-100 PBS 1x, 3 times, 5 minutes. The coverslips were incubated with the primary NF-κB p65 antibody (rabbit) (*Invitrogen*) at a 1:1000 dilution, overnight at 4°C with agitation. The coverslips were then washed 3 times, 5 minutes, with 0.05% Triton X-100 PBS 1x and were incubated with the secondary antibody, Alexa Fluor 532 anti-Rabbit IgG (*Life Technologies*) at a 1:500 dilution for 30 minutes. The coverslips were then washed with 0.05% Triton X-100 PBS 1x, 3 times, 5 minutes, with agitation. Next, the cells were quickly put through DAPI staining (4'6-diamidino-2-phenylindole) and the washing step was once again repeated. At the end the cells were fixed with 4% PFA for 15 minutes and then quickly washed two times with 0.05% Triton X-100 PBS 1x. The final step of this protocol required the coverslips to be assembled with the slides resorting to 4 μL of VectaShield mounting medium and then sealed with nailpolish. The resulting images were recorded with the 405-nm (DAPI) and 532-nm (NF-κB p65 subunit) laser lines of Leica TCS confocal microscope and processed with Image J software.

8. Statistical Analysis

Statistical analysis was performed with the Microsoft Excel program. The values were expressed as mean ± standard deviation. Ratio and proportion comparisons were made using a two tailed student t-test. Data statistically significant was accepted with a $-p < 0.05$ value.

III. RESULTS

Part 1

Role of activation of NF-κB canonical pathway on NIS expression

As stated above, TNF-α is a well-defined activator of the NF-κB pathway. In a previous study from the host lab, the overexpression of RAC1b, which is also a potent activator of NF-κB, was shown to be inversely correlated with NIS expression levels in thyroid follicular cell derived carcinomas (Faria *et al*., 2017b). Moreover, the ectopic overexpression of RAC1b in PCCL3 cells was shown to induce the downregulation of NIS mRNA expression (unpublished data). These studies led us to hypothesize that the activation of the NF-κB pathway could be associated with downregulation of NIS expression.

TNF-α was previously reported to induce a negative impact on NIS expression by downregulating NIS mRNA levels up to 70% in FRTL-5 rat thyroid cells (Spitzweg *et al.*, 1999). In this study, we aimed to confirm the effect of TNF-α on NIS expression in a different cellular model and to test whether this effect was dependent of NF-κB activation. We selected as an experimental model the rat thyroid follicular, non-neoplastic, PCCL3 cell line representative of a TSH-regulated system, previously shown to be responsive to TSH in what concerns NIS expression and iodide uptake. Cells were serum-starved for 24h before TSH stimulation (for additional 24h). Next, cells were treated with TNF-α for 1 or 24h. The effects on NIS mRNA levels were accessed by qRT-PCR (Figure III.1.).

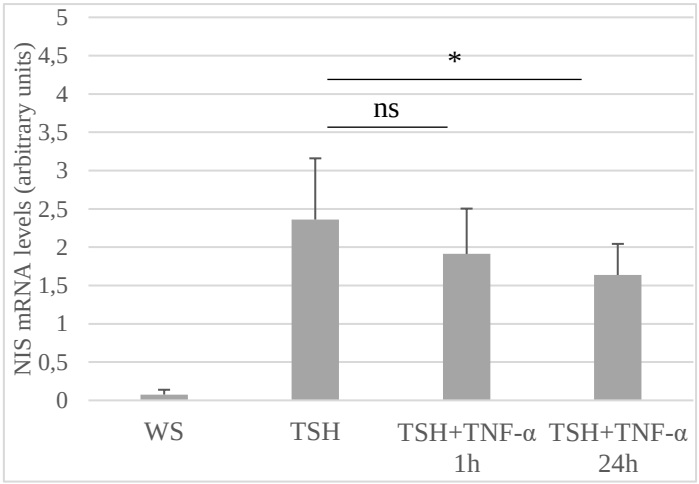

Figure III.1. Effect of TNF-α on NIS transcriptional regulation in PCCL3 cells. The levels of NIS expression were analysed by RT-qPCR. Cells were treated with TNF-α for 1h or 24h in the presence of TSH. Data are presented as the mean ± SD of at least three independent experiments. p-values were calculated comparing TSH alone with TNF-α treatments, using an unpaired Student's t-test. *p≤0.05 **p≤0.01 ***p≤0.001. ns: not statistically significant, ws: without stimulation.

Our data confirmed that TNF-α led to a downregulation of TSH-induced NIS expression that reached a statistically significant decrease with a 24h treatment. Then we asked whether this effect was only observed in the presence of TNF-α or if it could also occur upon stimulation of the cells with other NF-κB activators. In fact, it has been reported that LPS stimulation, by acting through NF-κB p65 subunit, exerts the opposite effect on NIS expression leading to its upregulation (Nicola *et al*., 2010). Since the activation of the canonical NF-κB pathway is triggered by both TNF-α and LPS stimuli, we wondered if the different outcomes could be a consequence of the differences in the upstream events, namely, in the ligand-receptor binding.

In this line of thought, we used PMA as a control (Figure III.2.), since it is a drug that acts independently of ligand-receptor specificity. It activates the signal transduction protein kinase C (PKC), a key player in the NF-κB pathway. PMA was described as a negative regulator of NIS protein function by reducing iodide uptake in human breast cancer cells and in rat pheochromocytoma cells ectopically expressing human NIS (Holden *et al*., 2008; Jung *et al*., 2008). However, no effects on NIS mRNA expression have been reported so far.

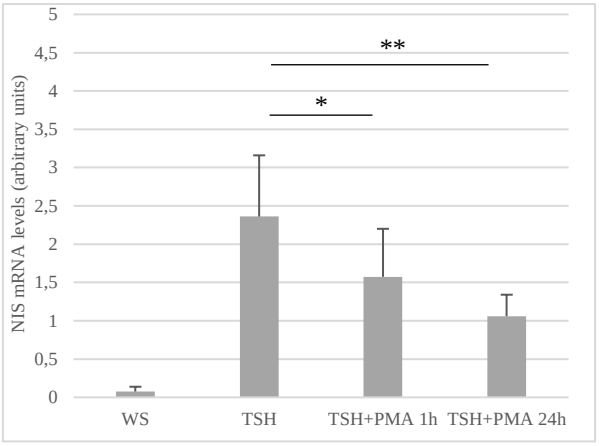

Figure III.2. Effect of PMA on NIS transcriptional regulation in PCCL3 cells. The levels of NIS expression were analysed by RT-qPCR. Cells were treated with PMA (1h and 24h) in the presence of TSH. Data are presented as the mean ± SD of at least three independent experiments. p-values were calculated comparing TSH alone with PMA treatments, using an unpaired Student's t-test. *p≤0.05 **p≤0.01 ***P≤0.001. ns: not statistically significant, without stimulation.

We observed that PMA had a negative impact on NIS mRNA expression similarly to that observed for TNF-α, suggesting that different triggers of NF-κB activation can lead to the same NIS expression outcome. Our next step was to test whether this effect was, in fact, occurring also through the canonical NF-κB pathway, as described for LPS. With this purpose, we used BMS-345541 (Figure III.3.), a highly selective inhibitor of IKK (Burke *et al*., 2002). Inhibition of IKK will hinder the phosphorylation of IκB-α (which is mandatory for its subsequent ubiquitination and proteolysis) which in turn will prevent the release of the p65 NF-κB subunit and its translocation from the cytoplasm to the nucleus, thereby blocking NF-κB-dependent transcription (Gilmore, 2006).

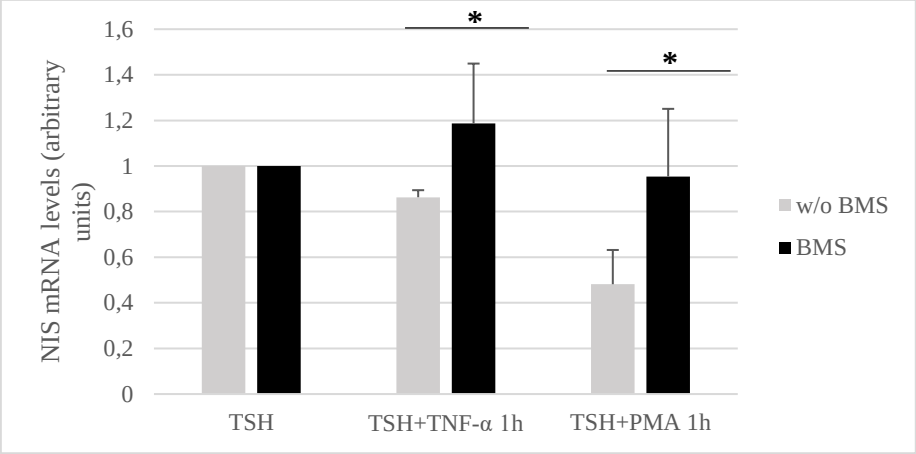

Figure III.3. Effect of TNF-α, PMA and BMS-345541 on NIS transcriptional regulation in PCCL3 cells. The levels of NIS expression were analysed by RT-qPCR. Cells were treated with TNF-α (1h) and PMA (1h) in the presence of TSH and in the presence or absence of BMS-345541 (6h). Data are presented as the mean ± SD. NIS mRNA levels in each condition were normalized to that of TSH stimulation in the absence of BMS-345541. Comparisons were made using a one-tailed Student's t-test. *p < 0.05 **p < 0.01 ***p < 0.001.

We observed that in the presence of BMS-345541 the decreased levels of NIS expression, induced by both TNF-α and PMA were restored to that of TSH stimulation alone. This suggests that both TNF-α and PMA downregulate the expression of NIS through the activation of the canonical NF-κB pathway, contrary to what is described for LPS.

In order to monitor the activation of the canonical NF-κB pathway, we used a NF-κB transcriptional readout by measuring the increase in IκB-α mRNA levels upon TNF-α and PMA stimulation (Figure III.4.). Besides being a NF-κB repressor, IκB-α is also a target gene of NF-κB transcriptional activity, generating a negative feedback loop. Therefore, one of the early events resulting from canonical NF-κB activation is the increase of IκB-α mRNA levels (Di *et al*., 2012). In fact, it was previously shown that IκBα mRNA levels perfectly correlate with the activation of NF-κB in several cellular models, indicating that quantification of IκBα is a valid readout of NF-κB activation (Bottero *et. al*, 2003).

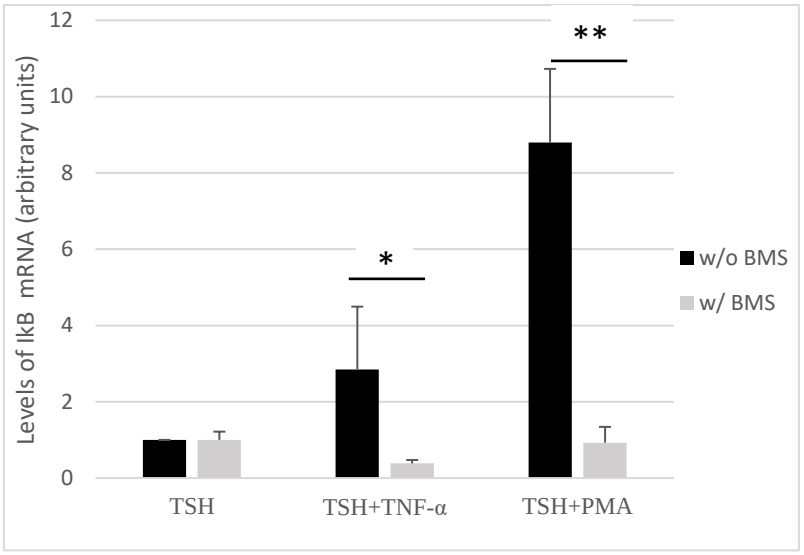

Figure III.4. Effect of TNF-α, PMA and BMS-345541 on IκB-α transcriptional regulation in PCCL3 cells. The levels of IκB-α mRNA expression were quantified by RT-qPCR. Cells were treated with TNF-α (1h) and PMA (1h) in the presence of TSH and in the presence and absence of BMS-345541 (6h). Data are presented as the mean ± SD. NIS mRNA levels in each condition were normalized to that of TSH stimulation in the absence of BMS-345541. Comparisons were made using a two-tailed Student's t-test. *p < 0.05 **p < 0.01 ***p < 0.001.

We observed that, in the absence of BMS-345541, IκB-α mRNA levels increase upon stimulation with TNF-α and PMA, consistent with NF-κB canonical pathway being activated. Even though both drugs activate NF-κB, PMA has a much stronger effect compared to TNF-α. In the presence of BMS-345541, the levels of IκB-α decreased to levels similar to that of the control.

To clarify that PMA and TNF-α were in fact inducing the nuclear translocation of the NF-κB p65 subunit, we addressed this matter by immunofluorescence followed by confocal microscopy (Figure III.5.). In this experiment, cells were subjected to serum starvation for 24h and then stimulated with TSH for 24h.

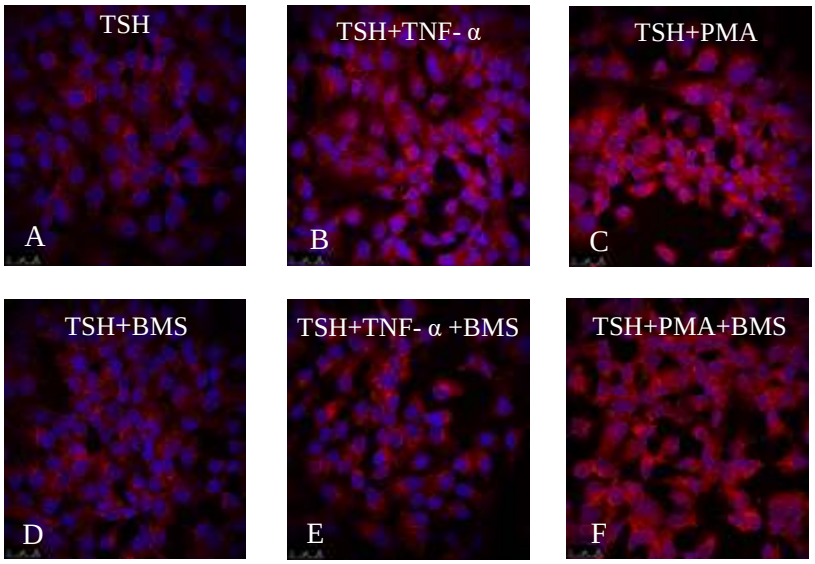

Figure III.5. Effect of TNF-α and PMA on p65 nuclear translocation in PCCL3 cells. Cells were treated with TNF-α (1h) and PMA (1h) in the presence of TSH and in the presence and absence of BMS-345541 (6h). Images of confocal microscopy of immunofluorescence staining of the anti-p65 antibody (red); nuclei were stained with DAPI (blue).

We observed that, in the presence of TSH and upon stimulation with TNF-α (B), there is a clear increase in the nuclear staining of NF-κB p65 subunit, compared to the control without TNF-α (A). When BMS-345541 was added to the cells treated with TNF-α (E), the nuclear staining of the p65 subunit decreased substantially. Similar effects can be seen in cells stimulated with PMA (C, F), except that, compared to TNF-α condition, BMS-345541 induced only a modest decrease in p65 nuclear accumulation. Nevertheless, this data confirms that both TNF-α and PMA promote the nuclear translocation of the NF-κB p65 subunit.

Part 2

Effect of PI3K/Akt/mTOR inhibition on NIS mRNA expression

The role of PI3K/Akt/mTOR pathway on NIS expression has been addressed in several studies, mostly in rat normal thyroid TSH-responsive cell models. Activation of this pathway has been reported to be associated with NIS downregulation. Some of the studies resorted to the use of the chemicals LY294002 and Rapamycin which inhibited PI3K and mTOR, respectively (Kogai *et al.*, 2008; Liu *et al*., 2012; de Souza *et al*., 2010; Platinga *et al*., 2014). However, from our knowledge, the effect of dual inhibition of this pathway on NIS expression has not yet been tested. In addition, data concerning the impact of PI3K/Akt/mTOR pathway on NIS expression in thyroid cancer cell models is still scarce and further studies are needed.

The dual inhibition of PI3K/Akt/mTOR pathway was carried out through the use of the chemical inhibitor BEZ-235, which reduces PI3K and mTOR kinase activity by competitive binding to their ATP-binding site (Lin *et al*., 2012). Additionally, aiming at the dual inhibition of this pathway, we also tested the combined action of LY294002 and Rapamycin, chemical inhibitors of PI3K and mTOR, respectively.

PCCL3 cells were serum-starved for 24h and then placed in stimulation medium (in the presence of FBS and TSH) for 24h (Figure III.6.).

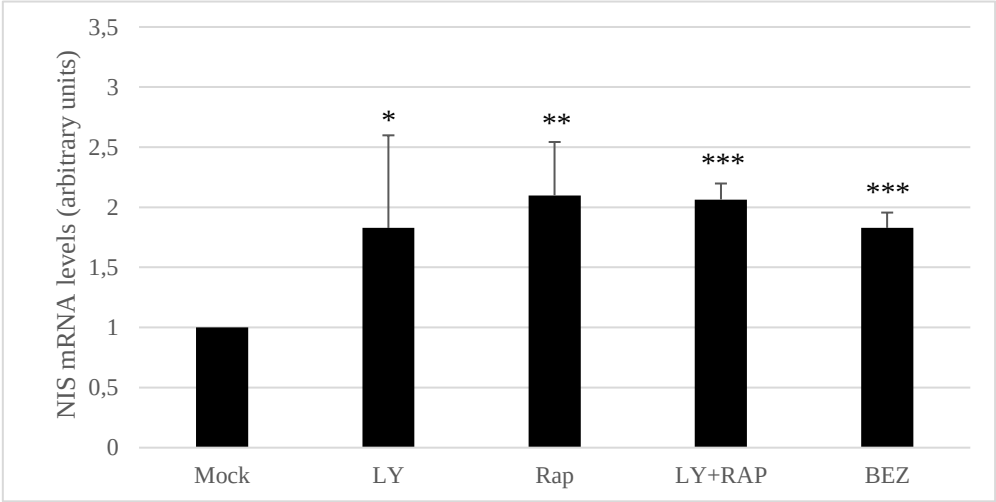

Figure III.6. Effect of LY294002, Rapamycin and BEZ-235 on NIS transcriptional regulation in PCCL3 cells. The levels of NIS expression were analysed by RT-qPCR. Cells were treated with LY294002 (6h, LY), Rapamycin (6h, Rap) and BEZ-235 (6h, BEZ) in the presence of FBS. Data are presented as the mean ± SD. NIS mRNA levels in each condition were normalized to the mock control (treatment with vehicle DMSO in the absence of inhibitors). Comparisons were made using a one-tailed Student's t-test. *p < 0.05 **p < 0.01 ***p < 0.001.

In the presence of serum, treatment of cells with LY294002, rapamycin or a combination of both inhibitors led to the increase of NIS mRNA by nearly two-fold compared to the control. A similar effect was observed after treatment with BEZ-235, the dual inhibitor of PI3K and mTOR.

To understand whether the effect of PI3K and mTOR inhibitors on NIS expression was a result of their impact on PI3K/Akt/mTOR pathway activity or whether these inhibitors were directly acting on the central pathway for TSH-induced NIS expression (TSH-cAMP-PKA), we reproduced the previous assay, in the presence of TSH stimulus, but in serum-depleted cells (to downregulate PI3K/Akt/mTOR signaling) (Figure III.7.). In this experiment, the increment in NIS expression was much less evident then the one observed in the presence of serum, suggesting that modulation of PI3K/Akt/mTOR signaling has a relevant impact on NIS expression.

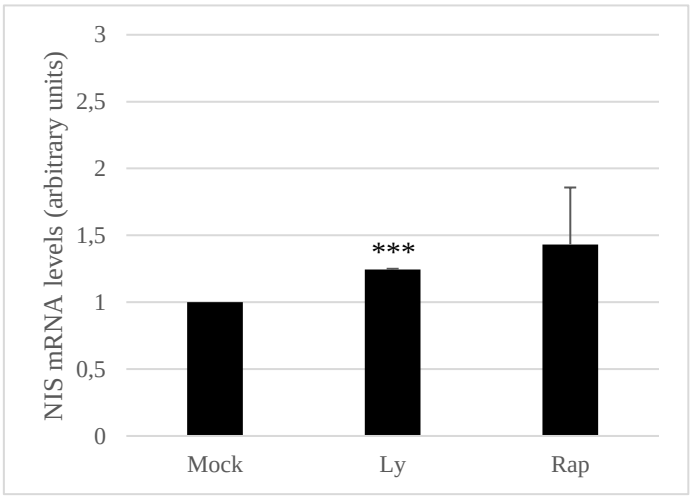

Figure III.7. Effect of LY294002 and Rapamycin on NIS transcriptional regulation in PCCL3 cells. The levels of NIS expression were analysed by RT-qPCR. Cells were treated with LY294002 (6h, LY) and Rapamycin (6h, Rap) in the absence of FBS. Data are presented as the mean ± SD. NIS mRNA levels in each condition were normalized to that of mock control (treatment with vehicle DMSO in the absence of inhibitors). Comparisons were made using a one-tailed Student's t-test. *p < 0.05 **p < 0.01 ***p < 0.001.

Next, we tested the effect of PI3K/Akt/mTOR inhibition in K1 cells, a human PTC-derived cell line that harbors a PI3K-activating mutation. Since NIS expression levels in this cell line are very low, we used it as a model to study the effect of the inhibition of the PI3K pathway on NIS expression. It has been previously shown that NIS expression levels increase in K1 cells after treatment with perifosine, an Akt inhibitor, and with temsirolimus, an mTOR inhibitor (an ester analog of rapamycin) in combination with suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor (Hou *et al*. 2009). However, from our knowledge, the increase of NIS expression in K1 cells by direct inhibition of PI3K or using a double inhibitor of PI3K/mTOR has never been attempted.

Thus, K1 cells were starved for 24h and then treated with LY294002, Rapamycin or BEZ-235 (in the presence of FBS) for an additional 24h (Figure III.8.).

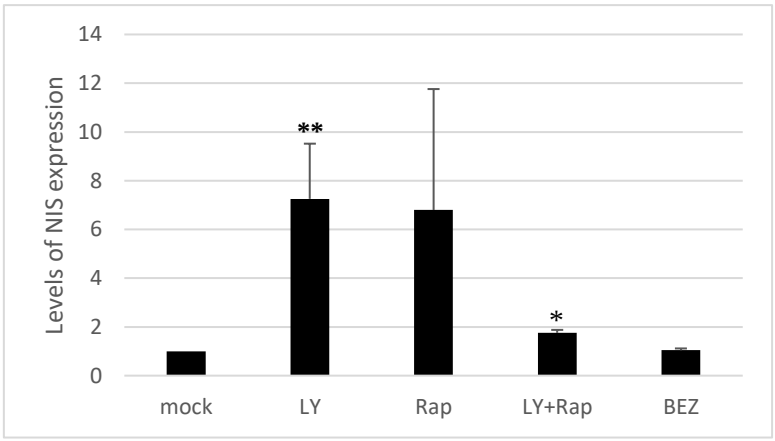

Figure III.8. Effect of LY294002, Rapamycin and BEZ-235 on NIS transcriptional regulation in K1 cells. The levels of NIS expression were analysed by RT-qPCR. Cells were treated with LY294002 (6h, LY), Rapamycin (6h, Rap) and BEZ-235 (6h, BEZ) in the presence of FBS. Data are presented as the mean ± SD. NIS mRNA levels in each condition were normalized to that of mock control (treatment with vehicle DMSO in the absence of inhibitors). Comparisons were made using a one-tailed Student's t-test. *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$.

We observed that treatment of K1 cells with either LY294002 or Rapamycin, greatly increased the transcriptional expression of NIS, although, statistical significance was not reached with Rapamycin. When LY294002 and Rapamycin were combined, an about two-fold increase in NIS expression was observed. This impact, however, was not as drastic as when the drugs were added separately. Of note, the PI3K/mTOR dual inhibitor BEZ-235 induced no effect on the symporter expression.

Additionally, the effects of LY294002, Rapamycin and BEZ-235 on PI3K/Akt/mTOR activation status in PCCL3 and K1 cells were monitored by accessing by Western Blot the levels of phospho-S6 protein, a downstream effector of the mTOR pathway (Figure III.9.; Figure III.10.).

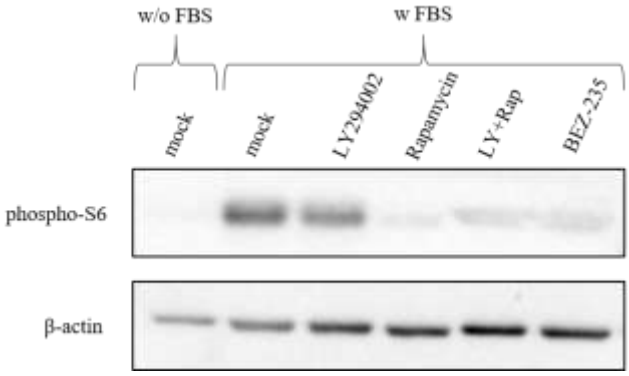

Figure III.9. Effect of LY294002, Rapamycin and BEZ-235 on phospho-S6 expression in PCCL3 cells. The levels of phospho-S6 protein expression were analysed by Western Blot. Cells were treated with LY294002 (6h), Rapamycin (6h) and BEZ-235 (6h) in the presence and absence of FBS. β-actin was used as a loading control.

We observed that the inhibition of PI3K with LY294002 had little effect on the PI3K/AKT/mTOR downstream effector phospho-S6, when comparing with the mock control. On the other hand, inhibiting this pathway either with Rapamycin alone or through PI3K/mTOR dual inhibition with LY294002+Rapamycin or BEZ-235, greatly decreased the expression of phospho-S6.

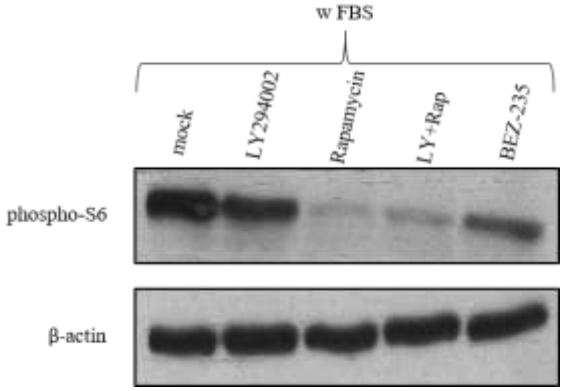

Figure III.10. Effect of LY294002, Rapamycin and BEZ-235 on phospho-S6 expression in K1 cells. The levels of phospho-S6 protein expression were analysed by Western Blot. Cells were treated with LY294002 (6h), Rapamycin (6h) and BEZ-235 (6h) in the presence and absence of FBS. β-actin was used as a loading control.

Similar effects were observed in K1 cells. Inhibiting the PI3K with LY294002 has a little impact on the expression of phospho-S6. Inhibition of the pathway with Rapamycin alone or with LY294002+Rapamycin greatly decreases phospho-S6 expression. However, inhibiting the pathway with BEZ-235 has a low impact on phospho-S6 expression, contrary to what was observed in PCCL3 cells.

IV. DISCUSSION

Thyroid cancer is the most common endocrine malignancy and its incidence has been increasing worldwide over the past decades. Most of thyroid cancers originate from thyroid follicular epithelial cells, being the well-differentiated forms the most frequent (Muro-Cacho *et al*., 2000; Nifikorov *et al*., 2011; Bhaijee *et al*., 2011). Most of these differentiated thyroid cancers retain the functional expression of NIS, allowing for the use of RAI as a systemic treatment to eliminate mestastases after total thyroidectomy (Castro et al., 2001; Wapnir *et al*., 2003). However, there is still a significant number of patients with advanced forms of thyroid cancer that lose the ability to respond to RAI therapy, greatly reducing their survival rates, since no efficient therapeutic alternatives are available (Durante *et al*., 2006).

Transcriptional and post-transcriptional regulation of NIS expression involves different molecular players and signalling pathways that are also implicated in tumour progression and agressiveness. In this work, we explored the impact of the PI3K/mTOR and NF-κB signalling pathways on NIS expression. Understanding the mechanisms underlying NIS functional regulation may allow the identification of regulatory events that can be pharmacological targeted to enhance the iodide uptake efficiency and improve the response to RAI therapy in RAI-refractory thyroid cancers.

NF-κB is a family of transcription factors responsible for regulating many cellular processes such as inflammatory responses, cellular growth and cell death. It has also been described to have a role in thyroid cancer development. Studies from the host lab pointed out an association between the GTPase RAC1b and the NF-κB pathway as well as between RAC1b and NIS (Faria *et al*., 2017a; Faria *et al*., 2017b). In fact, it was shown that overexpression of RAC1b signaling has a significant role in PTC tumorigenesis by inducing cell death resistance through NF-κB activation (Faria et al., 2017a). Moreover, data collected by the host lab supports a role for RAC1b in inhibiting NIS expression, since its overexpression was found to be inversely correlated with NIS levels in tumors and in a cell line representative of normal thyroid folicula. (Faria el al 2017b, unpublished work). Taken together, these results raised the hypothesis that the activation of the NF-κB pathway could be also involved in the downregulation of NIS. Thus, our main focus was to decipher the interplay between the NF-κB activation and NIS expression.

Our first approach was to assess the effect TNF-α had in the presence of TSH on NIS expression levels in the PCCL3 cell line. We decided to use TNF-α since, based on previous studies, it was reported to have a pro-inflammatory action and to promote the activation of NF-κB (Baldwin, 1996). Our results showed that TNF-α exerted a statistically significant down-regulatory effect at 24h on NIS expression. Accordingly, Spitzweg *et al*. (1999) described that TNF-α had a down-regulatory effect of 70% on NIS mRNA expression in FRTL-5 cells. Even though these associations were made, the role the NF-κB pathway plays on the down-regulatory effect this cytokine has on NIS is still to be deciphered. LPS, for instance, was described to up-regulate NIS expression levels and iodide uptake in FRTL-5 cells through activation of the canonical NF-κB pathway (Nicola *et al*., 2010). The different outcomes for NIS expression on both situations raised the hypothesis that there could be different mechanisms acting upstream of the formation of NF-κB active dimers, namely those related with the ligand-receptor binding, since the canonical NF-κB pathway seems to be activated by both TNF-α and LPS. Therefore, we tested the effect on NIS expression induced by NF-κB activation triggered by PMA, since this drug acts through direct activation of PKC, independently of ligand-receptor specificity. We observed that PMA downregulates NIS mRNA expression in PCCL3 cells, inducing a similar effect to the one of TNF-α, which indicates that different activators of NF-κB can induce a similar result on NIS expression. PMA has been previously shown to be able to induce cannonical NF-κB-dependent transcription (Holden *et al*., 2008). Its ability to impact on NIS expression, at least at the transcriptional level, had not been yet elucidated. It is worth noting, however, that PMA was shown to impact the NIS protein, acting as a downregulator of NIS function and iodine uptake

in human breast cells and rat pheochormocytoma cells ectopically expressing human NIS (Jung *et al*., 2008).

Since other signaling pathways besides the NF-κB canonical pathway could also be modulating the expression of the NIS gene we analysed the impact of PMA and TNF-α on the NF-κB activation in the presence of BMS-345541, an allosteric inhibitor of the IKK complex, (responsible for IκB-α phosphorylation) that mainly acts on IKKβ subunit (linked with the canonical NF-κB pathway) (Burke *et al*., 2002). In the presence of BMS-345541 the levels of NIS expression were restored in the cells treated with TNF-α and PMA indicating that the dowregulatory effect of these compounds on NIS expression occcurs through activation of the cannonical NF-κB pathway. To monitor the transcriptional activation of the canonical NF-κB pathway we also measured the levels of IκB-α expression, since it is one of the first genes to be transcribed by NF-κB activation. In both assays, with TNF-α and PMA, the data suggested that these two factors activate the canonical NF-κB pathway. These results were supported by immunofluorescent acessment of the nuclear translocation of the p65-NF-kB subunit, which reveals that, when stimulated with PMA or TNF-α, the cells activated the NF-κB pathway. Overall, from these experiments, we observed that TNF-α and PMA have a similar effect on the downregulation of NIS expression at transcriptional level through the activation of NF-κB, which seems contrary to what is reported with LPS stimulation (Nicola *et al*. 2010). The fact that similar stimuli may lead to different outcomes (in this particular case, the canonical activation of NF-κB having opposite effects in NIS transcription) may be explained by the synergistic action of additional pathways that may play an important role in modulating the affinity of the NF-κB for different promoters. Thus, NF-κB-driven transcription may lead to different results depending on the overall signaling involved in its regulation.

Considering the potential of modulating signalling pathways associated with thyroid cancer to achieve an increase in NIS expression, we aimed to evaluate the effect of the simultaneous targeting of PI3K and mTOR, since PI3K-AKT-mTOR pathway was previously shown to have negative impact on NIS.

Previous studies have demonstrated that, in TSH stimulated thyroid cells, the activation of PI3K/mTOR pathway reduced NIS expression levels affecting the iodide uptake by these cells. The effect of this pathway on iodide uptake was studied by using the PI3K inhibitor LY294002 which was shown to greatly hamper iodide uptake in both FRTL-5 and PCCL3 cells (Kogai *et al.*, 2008). It was also observed that PAX8 expression in PCCL3 cells remarkably increased after treatment with LY294002 and that the removal of insulin, a PI3K stimulator, reduced the effect of LY294002 on NIS mRNA levels (Kogai *et al.*, 2008). Our results are consistent with what is already reported, since we observed an increase in NIS mRNA expression (both in the presence or absence of FBS) when cells were treated with LY294002. Another inhibitor we used to study the PI3K/mTOR pathway was Rapamycin, a mTORC1 inhibitor, whose role was studied in rat thyroid cell lines and in human thyroid cancer cell lines (8505C, TPC1, and BCPAP). It was reported that Rapamycin could increase NIS expression and function in these cell lines, except for TPC-1 (de Souza *et al*., 2010; Platinga *et al*., 2014). Again, our results were consistent with previous data, as we observed that NIS mRNA expression in PCCL3 increases upon treatment with Rapamycin, both in the presence and absence of FBS. This increase was even higher than the one observed in the presence of LY294002, possibly since it acts on an effector more downstream in the pathway. This drug, however, has the disadvantage of inhibiting only one of the two forms of the mTOR complex that may lead to treatment resistance. Consequently, other strategies have been explored, such as the use of dual inhibitors for mTORC1 and mTORC2 or multiple targeted therapy which involves the inhibition of mTOR and Akt or dual inhibition of PI3K/Akt and mTOR with BEZ-235 (Petrulea *et al.*, 2015). This drug reduces PI3K and mTOR kinase activity by competitive binding to their ATP-binding site ultimately inhibiting cell proliferation (Maira *et al*., 2008; Lin *et al.*, 2012). The effects of BEZ-235 in inhibiting cell proliferation were particularly notorious in ATC cell lines as well as in poorly differentiated FTC cell line (Lin *et al.*, 2012).

From our knowledge, no studies evaluating the effect of dual inhibition of PI3K and mTOR on NIS expression have been performed yet. With this purpose, we treated PCCL3 cells with BEZ-235 and observed an increase in NIS mRNA levels by nearly two-fold compared to the control. Since BEZ-235 has a similar effect to the other inhibitors studied, it apparently does not bring any advantage in what concerns the increase of NIS mRNA expression. Nevertheless, in the context of cancer therapeutic resistance, the use of this inhibitor may be of added value.

Next, we studied the effect of PI3K/mTOR inhibition on NIS expression in the K1 human PTC-derived cell line, a thyroid cancer cell model which has a PI3K-activating mutation, (Pilli *et al*. 2009) and expresses very low NIS mRNA levels. Both inhibitors, LY294002 and Rapamycin, induced a dramatic increase on NIS mRNA levels (although, statistical significance was not reached with Rapamycin), being able to restore its expression in these cells. Unexpectedly, the dual-inhibition of PI3K/mTOR by BEZ-235, exerted almost no effect on NIS mRNA expression. This may be due to the use of an amount of inhibitor not adequate for this cell type or to other particularities of this cell line, possibly associated with the overall cell signaling. Optimal experimental conditions should be assessed in future studies. Consistent with our results, a previous study reported that Akt and mTOR inhibitors were able to induce an increase in NIS transcript levels in K1 cells. However, in this study, the effect of these inhibitors was only achieved when combined with a histone deacetylase inhibitor (Hou *et al*. 2009).

Additionally, the effect of PI3K/mTOR inhibitors on the pathway activity was monitored by assessing the expression of phospho-S6 both in PCCL3 and K1 cell lines. The results in PCCL3 cells reveal that all the drugs tested, LY294002, Rapamycin and BEZ-235, were effective in inhibiting PI3K/mTOR pathway. Rapamycin was the most effective in downregulating the expression of phospho-S6, which is consistent with its impact on NIS expression. Of the three inhibitors, LY294002 had the least impact on phospho-S6, albeit being able to induce a relevant upregulation of NIS expression.

In the K1 cell line, the impact of PI3K/mTOR inhibitors was similar to the one observed in PCCL3 cells when using phospho-S6 as readout of pathway activation. However, even though BEZ-235 was shown to be able to inhibit the PI3K/mTOR pathway in K1 cells (still acting more efficiently than LY294002 as observed in PCCL3 cells) it does not seem to exert the same impact on phospho-S6 expression as it does in PCCL3 cells, which appear to be more sensitive to the effect of this inhibitor. This might be associated with the discrepancy observed between PCCL3 and K1 cell lines in the impact of this drug on NIS expression.

V. CONCLUSIONS AND PERSPECTIVES

In this project we aimed to contribute to the identification of signaling pathways that may lead to the enhancement of NIS expression levels.We studied the role of the NF-κB canonical pathway on the expression of NIS in a normal cell line derived of rat thyroid tissue. Additionally, we also investigated the role of the PI3K/mTOR pathways on NIS expression in the same rat cell line and in K1, a human thyroid derived cell line with very low expression of NIS and a constitutive PI3K activation.

The data obtained in this study supports a role of the NF-κB pathway in the regulation of NIS mRNA expression. Particularly, we observed in PCCL3 cells that TNF-α and PMA are able to downregulate NIS expression by activating the canonical NF-κB pathway. In addition, our data is consistent with previous work supporting the inhibitory role of the PI3K/mTOR pathway in the regulation of NIS. Nonetheless, from our knowledge, the effect of the combined inhibition of PI3K and mTOR on NIS expression has not yet been tested. We found, however, that dual-inhibition of the PI3K/mTOR pathways brought no advantage to the increase of NIS expression when compared with the single inhibition of PI3K or mTOR. Moreover, we were able to restore NIS expression in thyroid cancer cells with constitutive activation of PI3K through PI3K/mTOR pathway inhibition with LY294002 and Rapamycin.

In the future, it would be interesting to evaluate whether the pathways tested also have an impact on NIS protein levels or NIS subcellular localization, in both thyroid cancer and non-neoplastic cell models. Furthermore, it would be also relevant to study how the impairment of the NF-κB activity with BMS-345541 will interfere with its physiological roles and, if so, identifying targets that are preferentially activated in tumors compared to normal cells.

Understanding the regulatory mechanisms and signaling pathways behind NIS expression can contribute to the further identification of key regulatory elements that may be pharmacologically targeted for thyroid cancer treatment.

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VII. APPENDIX

Table VII.1. Experimental work solutions

Solutions	Reagents	Final concentration
10x PCR buffer	Tris-HCl	100 mM
	KCl	500 mM
	$MgCl_2$	15 mM
	Gelatin	0.1% (w/v)
1x PCR buffer	10x PCR buffer	1 mL
	H_2O	9 mL
	100 mM dNTP	20 µL of each dNTP (dATP, dCTP, dGTP, dTTP)
Sample buffer	Benzonase (*Sigma*)	1 U/µL
	$MgCl_2$	5 mM
	Tris-HCl pH 6.8	200 mM
	SDS	2%
	DTT	100 mM
	Glycerol	5%
	Bromophenol blue	0.1% (v/v)
SDS-PAGE resolving gel	Resolution buffer (Tris-HCl pH 8.8)	375 mM
	Acrylamide	10%
	SDS	0.1% (v/v)
	TEMED	0.05% (v/v)
	APS	1% (v/v)
SDS-PAGE stacking gel	Resolution buffer (Tris-HCl pH 8.8)	62.5 mM
	Acrylamide	4%
	SDS	0.1% (v/v)
	TEMED	0.05% (v/v)
	APS	1% (v/v)
Blot buffer pH 7.6	Tris	25 mM
	Glycine	192 mM
	SDS	0.03% (w/v)
	Methanol	5% (v/v)
Destain solution	Acetic acid	10% (v/v)
	Methanol	45% (v/v)
TBST solution pH 7.6	Tris-HCl	50 mM
	NaCl	150 mM
	Triton X-100	15 mM
ECL (solution 1)	Tris pH 8.8	100 mM
	Luminol	3.75 mM
	Cumaric acid	450 µM
ECL (solution 2)	Tris pH 8.8	100 mM
	Hydrogen Peroxide	0.1% (v/v)

Table VII.2. Thermocycling conditions for Taq polymerase PCR amplification of NIS rat, NIS human and IκB-α.

NIS rat			
Step	**Temperature °C**	**Time**	**Cycles**
Initial denaturation	95	5 min	1
Denaturation	95	1 min	26
Annealing	65	1 min	
Elongation	72	1 min	
Final Elongation	72	7 min	1

NIS human			
Step	**Temperature °C**	**Time**	**Cycles**
Initial denaturation	95	5 min	1
Denaturation	95	1 min	40
Annealing	64	1 min	
Elongation	72	1 min	
Final Elongation	72	7 min	1

IκB-α rat			
Step	**Temperature °C**	**Time**	**Cycles**
Initial denaturation	95	5 min	1
Denaturation	95	30 sec	27
Annealing	60	30 sec	
Elongation	72	30 sec	
Final Elongation	72	3 min	1

Table VII.3. Conditions for qRT-PCR.

Step	Temperature °C	Time	Cycles
Initial denaturation	95	2 min	1
Denaturation	95	15 sec	40
Annealing	60	30 sec	
Elongation	72	15 sec	