



UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Scienze Cardiologiche, Toraciche e Vascolari

SCUOLA DI DOTTORATO DI RICERCA IN: Scienze Mediche, Cliniche e Sperimentali

INDIRIZZO: “Metodologia Clinica, Scienze Endocrinologiche, Diabetologiche e Nefrologiche”

CICLO: XXVIII

Novel molecular mechanisms involved in the pathogenesis of GH-secreting pituitary adenomas

Direttore della Scuola: Ch.mo Prof. Gaetano Thiene

Coordinatore d'indirizzo: Ch.mo Prof. Roberto Vettor

Supervisore: Prof. Carla Scaroni

Dottorando: Dr. Denis Ciato

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Abbreviations

17-AAG	17-(Allylamino)-17-demethoxygeldanamycin
17-DMAG	17-(dimethylaminoethylamino)-17demethoxygeldanamycin
3MC	3-methylcholanthrene
ACTH	adrenocorticotroph hormone
ADP	adenosine diphosphate
AHR	aryl-hydrocarbon receptor
AHRE	aryl-hydrocarbon receptor-responsive element
AIP	AHR-interacting protein
ATP	adenosine triphosphate
BaP	benzo[a]pyrene
bHLH	basic helix-loop-helix
cAMP	cyclic nucleotide adenosine 3',5'-monophosphate
CDK	cyclin dependent kinase
CNS	central nervous system
CREB	cAMP response element-binding protein
CTD	carboxy-terminal domain

CYP	cytochrome P450
ED	endocrine disruptor
FSH	follicle-stimulating hormone
GA	geldanamycin
GH	growth hormone
GHRH	growth-hormone-releasing hormone
GPCR	G protein-coupled receptor
HSF	heat shock factor
HSP	heat shock protein
IGF	insulin-like growth factor
LH	luteinising hormone
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
NES	nuclear export sequence
NFPA	non-functioning pituitary adenoma
NLS	nuclear localization sequence
NTD	amino-terminal domain

OIS	oncogene-induced silencing
PCB	polychlorinated biphenyl
PDE	phosphodiesterase
PKA	protein kinase A
PRL	prolactin
PTTG	pituitary tumor-transforming gene
qPCR	real-time PCR
ROS	oxygen-reactive species
SA- β Gal	senescence-associated beta-galactosidase
SD	standard deviation
SRL	somatostatin receptor ligand
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TOMM	translocase of the outer membrane of mitochondria
TNF	tumor necrosis factor
TPR	tetratricopeptide
TSG	tumor suppressor gene
TSH	thyroid-stimulating hormone

WHO	world health organization
XRE	xenobiotic responsive element
β NF	beta-naphthoflavone

Abstract

Pituitary tumors usually show a particular benign nature, with a slow growth potential. Despite these characteristics, they can cause severe complications due to their excessive hormone secretion. Still, further investigations are needed to understand the molecular mechanisms implicated in their development in detail. AHRR (Aryl Hydrocarbon Receptor (AHR) Repressor) and HSP90 (Heat Shock Protein 90) are good candidates for playing a role in pituitary tumorigenesis due to their tumorigenic role reported in different types of tumors and their action in the metabolism of endocrine disruptors (EDs), in which AIP (Aryl Hydrocarbon Receptor Interacting Protein) is also implicated. AHR Repressor (AHRR) is the main regulator of AHR activity through a negative feedback mechanism, which impairment leads to tumor-promoting events. Accordingly, AHRR downregulation was reported in several malignancies including colon, breast and lung cancer and was correlated with a more malignant phenotype (e.g. resistance to apoptosis, increased cellular motility and angiogenic potential) in different tumor cell lines. Although several studies investigated the potential role of AHR signaling in pituitary tumorigenesis, so far no data has been available on the role of AHRR in pituitary tumorigenesis. Therefore, the primary aim of the present study was to evaluate the possible role of AHRR in influencing the tumor phenotype in the somato-lactotroph cell line GH3 in which AHRR was stably silenced or overexpressed. AHRR-silenced cells showed a less aggressive phenotype due to a decreased cell viability and to a more pronounced resistance to apoptosis-inducing stimulus in comparison to the control. Correspondingly, opposite results were obtained for the stable overexpression of AHRR. In addition, AHRR silencing increased senescence-associated beta

galactosidase (SA- β Gal) staining in accordance with a slow-growing phenotype observed at proliferation level, without having effect on cell cycle progression. No correlation was observed between AHRR mRNA expression in human pituitary adenomas and tumor aggressiveness, although GH-secreting tumors displayed a higher expression of AHRR in comparison to normal pituitaries.

The second part of this thesis was aimed at the role of HSP90 in the pathogenesis of GH-secreting tumors. In addition to the stabilization of AHR, this protein plays a pivotal role in maturation and stabilization of other proteins implicated in oncogenic signaling and cancer progression, and treatment with HSP90 inhibitors has recently shown promising results in pituitary corticotroph adenomas. Therefore, the function of HSP90 was investigated in GH-secreting pituitary adenomas. An intense HSP90 immunoreactivity was reported in 8 out of 25 GH-secreting pituitary tumors. In order to study the therapeutic potential of HSP90 inhibition in these tumors, the cell line GH3 was treated with different HSP90 inhibitors. The C-terminal HSP90 inhibitors novobiocin and KU174 dose-dependently decreased GH promoter activity, whereas only KU174 decreased GH secretory levels.

Conclusively, the data presented herein provide novel insights in the pathogenesis of GH-secreting pituitary adenomas. AHRR might have an oncogenic role by influencing tumor aggressiveness, as shown in the cellular model. Moreover, the results obtained in the second part suggest that HSP90 might have a role in the tumorigenesis of GH-secreting pituitary tumors and a potential for HSP90 C-terminal inhibitors in managing the excess of GH secretion.

Riassunto

I tumori ipofisari manifestano normalmente un fenotipo benigno, caratterizzato da un potenziale di crescita ridotto. Anche in assenza di effetti patogenetici dovuti all'espansione della massa tumorale nelle strutture adiacenti, possono causare severe complicazioni a causa della loro eccessiva secrezione ormonale. Studi approfonditi sono ancora necessari per riconoscere nuovi meccanismi molecolari implicati nel loro sviluppo. AHRR (Aryl Hydrocarbon Receptor (AHR) Repressor) e HSP90 (Heat Shock Protein 90), oltre ad avere un ruolo nella tumorigenesi in tessuti di differente origine, potrebbero potenzialmente essere coinvolti nello sviluppo dei tumori ipofisari per via della loro attività nel metabolismo di *Endocrine Disruptors* (EDs), in cui anche la proteina AIP (*Aryl Hydrocarbon Receptor Interacting Protein*) è implicata. AHRR è il principale regolatore dell'attività di AHR, e lo sbilanciamento di questo sistema di regolazione può avere effetti tumorigenici. Nella fattispecie, l'espressione ridotta di AHRR è stata descritta in tumori di differente origine anatomica tra i quali colon, seno e polmone; e sembra essere associata ad una maggiore aggressività del fenotipo tumorale (in termini di resistenza all'apoptosi, aumento della proliferazione e potenziale angiogenico) in differenti modelli cellulari. Nonostante diversi studi abbiano investigato il potenziale ruolo della via di segnale di AHR nei tumori ipofisari, non vi sono evidenze riguardo al ruolo specifico di AHRR. Scopo principale di questo lavoro di tesi è stato perciò valutare il possibile ruolo di AHRR nella patogenesi dei tumori ipofisari. La linea cellulare somatotropa GH3 è stata utilizzata come modello per generare cloni stabili in cui AHRR è silenziato o overespresso. Il silenziamento di AHRR ha portato alla manifestazione di un fenotipo meno aggressivo dovuto alla diminuzione della capacità proliferativa

e alla maggiore resistenza a stimoli apoptotici rispetto al controllo, mentre risultati opposti sono stati ottenuti overesprimendo stabilmente AHRR. È stato inoltre evidenziato come il silenziamento di AHRR abbia indotto la manifestazione di un fenotipo più senescente rispetto al controllo, in correlazione con la riduzione della proliferazione cellulare. Non sembra però che ciò coinvolga un cambiamento sulla distribuzione del ciclo cellulare. Inoltre, forse a causa del numero ridotto di campioni analizzati, la valutazione quantitativa dell'espressione a livello dell'mRNA di AHRR nei tumori ipofisari non sembra essere in relazione con l'aggressività tumorale, anche se nel sottogruppo adenomi GH-secernenti vi sia una maggiore espressione rispetto a campioni di ipofisi normali.

La seconda parte di questo lavoro di tesi ha avuto come scopo la valutazione del ruolo di HSP90 nella patogenesi dei tumori ipofisari. Oltre alla stabilizzazione di AHR, questa proteina svolge un ruolo fondamentale nella maturazione e nella stabilizzazione di altre proteine implicate nella trasformazione e nella progressione tumorale, e la sua inibizione specifica ha recentemente dimostrato risultati promettenti negli adenomi ACTH-secernenti. Sulla base di queste premesse, la valutazione del ruolo di HSP90 è stata estesa ai tumori GH-secernenti. L'analisi immunohistochimica effettuata su adenomi GH secernenti ha evidenziato una intensa immunoreattività di HSP90 in 8/25 casi. Visto il potenziale coinvolgimento di HSP90 in questa tipologia di adenomi ipofisari, la linea cellulare GH3 è stata utilizzata come modello per investigare il potenziale terapeutico di differenti inibitori di HSP90. Nella fattispecie, il trattamento con gli inibitori C-terminali di HSP90 novobiocina e KU174 ha provocato una diminuzione dose-dipendente dell'attività promotoriale del GH, mentre solo KU174 ha significativamente diminuito la secrezione di GH.

In conclusione, i risultati ottenuti forniscono evidenze funzionali sul possibile ruolo oncogenico di AHRR nell'ipofisi, influenzando sul fenotipo aggressivo nel modello cellulare utilizzato. Abbiamo inoltre dimostrato il potenziale ruolo di HSP90 nella patogenesi dei tumori ipofisari GH-secerenti, e il trattamento con inibitori C-terminali di HSP90 potrebbe risultare efficace nel diminuire l'eccessiva produzione e secrezione di GH.

1. Introduction

1.1 The pituitary gland

1.1.1 Anatomy

The pituitary gland is one of the master regulators of the endocrine system, including the thyroid gland, adrenal glands, ovaries and testes under its control. It situates in the *sella turcica* of the sphenoid, below the optic chiasma, and is connected with the *tuber cinereum* of the hypothalamus through the pituitary peduncle. The pituitary gland consists of two major parts fused together, but with different functions, the adenohypophysis and the neurohypophysis, both connected to different neuro-secreting regions of the hypothalamus¹ (Figure 1.1). The neurohypophysis is of neuro-ectodermic origin and is formed by the pituitary peduncle and the posterior lobe. The peduncle is constituted of the median eminence (or infundibulum) and the infundibular trunk. The posterior lobe contains pituicytes, specialized glial cells assisting in the storage and release of the hormones¹. The adenohypophysis is of ectodermic origin and is divided in the anterior lobe, and in the intermediate lobe, very reduced in mature human pituitary gland in compared to in human fetus or most animals. The anterior lobe is made of a highly vascularized stromal layer, in which parenchymal and endocrine epithelial cells are included. Specialized cells of the pituitary, especially in the adenohypophysis, produce and secrete different hormones, from which these cells take their names¹:

-somatotroph cells, producing the somatotroph hormone or growth hormone (GH), which acts on metabolism and cellular division or stimulation of the liver production of insulin-like growth factor-1 (IGF-1), causing a general increase in the dimensions of the organism;

-adrenocorticotroph cells, producing the adrenocorticotroph hormone (ACTH), which among multiple other roles stimulate the secretion of cortisol from the adrenal cortex;

-lactotroph cells, producing the lactotroph hormone or prolactin (PRL), controlling mammary development, which among others regulate the action of gonadotropins on their target organs in men and women.

-gonadotroph cells, producing gonadotropins (follicle-stimulating hormone, FSH, and luteinising hormone, LH), which act in the development of gonads in women and men;

-thyrotroph cells, producing the thyroid-stimulating hormone (TSH), which stimulates the secretion of thyroid hormones¹.

-cells from the neurohypophysis in the posterior lobe secreting oxytocin and vasopressin or antidiuretic hormone (ADH), which are produced in the neuro-secreting neurons of the supraoptic and paraventricular nuclei of the hypothalamus. These hormones stimulate the contraction of the muscles of the uterus during and after the delivery and the reabsorption of water in the distal and collecting ducts of the kidney respectively^{2,3}.

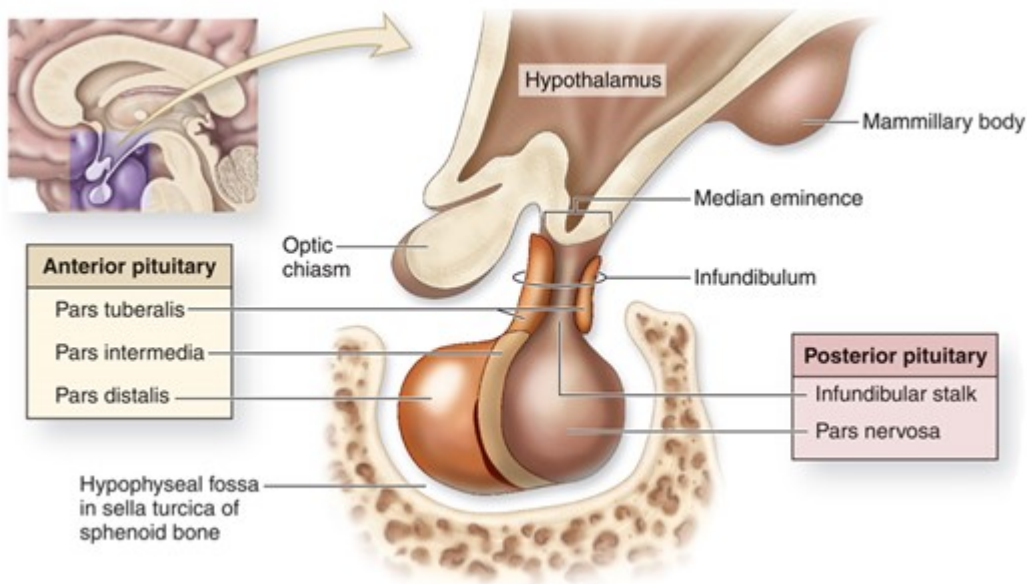


Figure 1.1: Schematic representation of the pituitary gland. Adapted from <https://sites.google.com/a/wisc.edu/neuroradiology/anatomy4>.

1.1.2 Regulation of hormone secretion

Pituitary hormone secretion is regulated through three different levels of control. Hypothalamic control is mediated by adenohipophysiotropic hormones secreted into the portal system that impact on anterior pituitary cell surface receptors, which are G protein-coupled receptors (GPCRs) specific for releasing and inhibiting hormones. Peripheral hormones also participate in mediating pituitary function, mainly through negative feedback regulation of trophic hormones by their respective target hormones. Intrapituitary paracrine and autocrine factors like cytokines or growth factors are also acting for regulating pituitary cells development and function. The result of the regulation of this complex system is the temporal control of the secretion of the pituitary hormones (ACTH, GH, PRL, TSH, FSH and LH) through the cavernous sinus, the petrosal veins and the systemic circulation (Figure 1.2)⁵. Because most data concern acromegaly in this thesis, the next paragraphs will be focused on the regulation of GH and PRL synthesis.

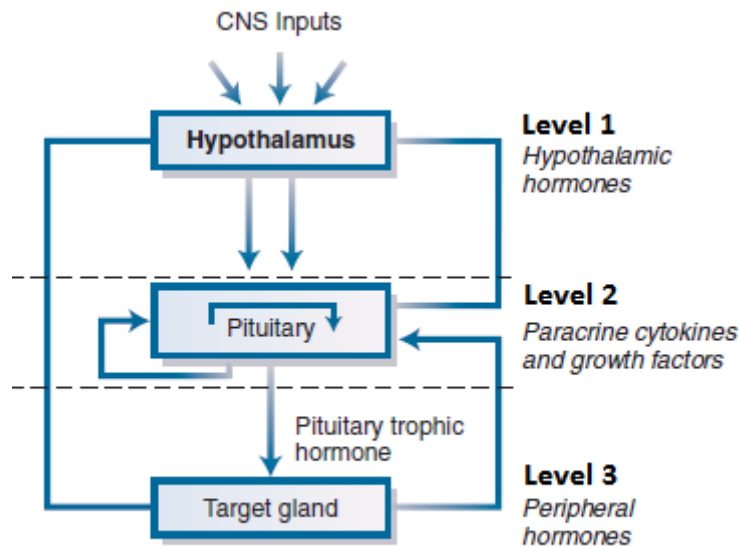


Figure 1.2: Regulation of anterior pituitary hormone secretion at different levels. Level I: Hypothalamic hormones impact on the receptors on the respective target cells. Level II: Intrapituitary growth factors and cytokines regulate differentiation and function of pituitary cells by paracrine and (autocrine) action. Level III: Peripheral hormones exert negative feedback inhibition of synthesis and secretion of pituitary hormones. CNS: central nervous system. Adapted from Ray et al., 1997 and Melmed et al., 2001^{5,6}.

1.1.2.1 Regulation of GH synthesis

GH has a pivotal role in the organism in promoting organ and soft tissue growth. It is secreted by somatotroph cells within the lateral wings of the anterior pituitary gland, and its synthesis is controlled by a complex regulatory mechanism. GH production and secretion is regulated by hypothalamic growth-hormone-releasing hormone (GHRH), ghrelin and somatostatin; IGF-1 inhibits GH secretion by a direct effect on the somatotroph cells and indirectly by stimulating the expression of somatostatin, which in turn inhibits GH secretion^{7,8}. The expression of the GH gene is mainly controlled by a process in which elevated intracellular levels of cyclic adenosine monophosphate (cAMP) activate protein kinase A (PKA)^{9,10}. In the

human GH promoter there are two cAMP-responsive elements (CREs); moreover, in the promoter region of the gene there are two sites for the POU domain, class 1, transcription factor 1 (PIT-1); all of these sites are essential for cAMP responsiveness and for GH promoter activity *in vitro* and *in vivo*¹¹⁻¹³. When secreted and bound to its specific receptor, GH exerts some of its physiological effects by activating the MAPK/ERK pathway and JAK-STAT pathway¹⁴.

1.1.2.2 Regulation of PRL synthesis

Prolactin is synthesized and secreted by lactotroph cells of the anterior pituitary gland. Primarily known for its role in enabling mammals, usually females, to produce milk, it also controls a variety of behaviors and even plays a role in homeostasis¹⁵. Transcription of the PRL gene is regulated by two independent promoter regions, consisting in one that directs the pituitary-specific expression and another one, more upstream, that is responsible for extrapituitary expression. Its secretion is regulated by endocrine neurons in the hypothalamus¹⁶; among these, a very important role is exerted by dopamine, which inhibits the basal high-secretory tone of lactotroph cells¹⁷. When synthesized, prolactin interacts with specific receptors present in mammary glands, ovaries, pituitary glands, heart, lung, thymus, spleen, liver, pancreas, kidney, adrenal gland, uterus, skeletal muscle, skin, and areas of the central nervous system. Binding with the receptor causes its dimerization, resulting in the activation of the mitogen-activated-protein kinases (MAPK) pathway, the Src kinase and the JAK-STAT pathway¹⁸.

1.2 Pituitary Tumors

Pituitary tumors comprehend nearly 15% of intracranial neoplasms¹⁹. They can arise from each type of hormone-secreting cells of the adenohypophysis, and the

majority of them are of monoclonal origin. Their typical hallmarks are excessive cellular proliferation and over-secretion of their respective hormones, but they can also be functionally silent⁵. Although generically classified as benign, they can cause severe health complications due to the aberrant hormone secretion and the invasion of surrounding structures²⁰.

1.2.1 Classification of pituitary tumors

Pituitary tumors are classified according to: a) the clinical manifestation of the hormonal activity *in vivo*; b) immunohistological hormone staining; and c) tumor size and degree of local invasion. The most recent update on the classification of pituitary tumors dates to the World Health Organization (WHO) Classification of 2004, in which novel putative oncogenes and tumor suppressor genes have been identified and more pathological markers were postulated to predict the biological behavior of pituitary adenomas^{21,22}.

a) Clinical hormone-secreting types are generally classified as:

- GH-secreting adenomas or somatotrophinomas
- PRL-secreting adenomas or prolactinomas
- GH-and PRL-secreting adenomas or mammosomatotroph adenomas
- ACTH-secreting adenomas or corticotrophinomas
- TSH-secreting adenomas or thyrotrophinomas
- FSH-and LH-secreting adenomas or gonadotrophinomas
- Non-functioning adenomas (NFPAs).

PRL-secreting adenomas have the highest prevalence (40-45%) among all types of pituitary tumors. Lesser common types of pituitary adenomas are in decreasing order NFPAs (30%), somatotrophinomas (15-20%), corticotrophinomas (5-15%),

thyrotrophinomas and FSH-LH adenomas (<1%)²³⁻²⁵. Mixed types of tumors can also occur, the most common combination being GH and PRL (5.2%)^{21,26,27}.

b) Histological classification distinguishes the different types of pituitary adenomas according to their reaction to staining into:

- acidophilic (GH and/or PRL-producing tumors)
- basophilic (ACTH, TSH and FSH/LH secreting tumors)
- chromophobic (hormonally inactive tumors).

c) The classification of tumors according to size and invasion classifies pituitary tumors in different grades:

- Grade I refers to microadenomas, with diameter <10mm and contained in the pituitary fossa
- Grade II refers to macroadenomas, with diameter >10mm that may have suprasellar extension but no invasion in the surrounding structures
- Grade III for locally invasive tumors
- Grade IV refers to large invasive tumors that can involve the hypothalamus and cavernous sinuses.

1.2.1.1 Aggressive pituitary adenomas

Pituitary tumors are generally benign and non-invasive. A significant number of pituitary tumors (between 45 and 55%) display features of invasiveness while maintaining benign behavior²⁸. Pituitary tumors are defined as malignant (pituitary carcinomas) when showing cerebrospinal or systemic metastasis; they are rare, with an incidence of 0.2% of symptomatic pituitary tumors²⁸. Aggressive pituitary tumors take their place between benign and malignant adenomas with a rather distinct

clinical behavior, invasion of the anatomical structures surrounding the pituitary, tendency to withstand medical treatment and early postoperative recurrence²⁹. In 2004 the WHO published a novel classification system for pituitary tumors based upon immunohistochemistry for the detection of secretory products and other ultrastructural features. The term “atypical tumors” was introduced in this categorization. It includes tumors with “atypical” morphological features suggestive of an “aggressive behavior”, substantiated by the presence of invasive growth, high mitotic index, a Ki67 labelling index >3% and extensive nuclear staining for p53³⁰. This classification is the first attempt at identifying tumors with a distinctive behavior compared to benign pituitary adenomas, but it displays an important weak spot by lacking clear consensus regarding the definition of invasive growth³¹. Due to the lack of a clear consensus on pituitary tumor aggressiveness, a small spotlight is put on histological and radiological parameters in the next paragraphs.

1.2.1.1.1 Histological parameters: MIB-1 and p53 IHC staining

The histological parameters for identifying the behavior of pituitary tumors take into account different factors that play a role in proliferation of pituitary tumor cells. MIB-1 monoclonal antibody is the most commonly used one for the detection of Ki67 staining and evaluation in paraffin-embedded tissue specimens of pituitary adenomas, with a reasonable correlation with pituitary adenomas growth rate^{32,33}. High levels of the tumor suppressor p53 have been found in many cancers and have been considered as a marker for tumor atypicalness in the pituitary. However, there is still some debate about the value of these markers as predictors of aggressive behavior of pituitary adenomas in daily practice. Those tumors that exceed the WHO cut-offs for Ki67 labelling index and present intense immunoreactivity for p53 are uncommon even in tumors with postsurgical progression, and there is no clear

correlation between immunohistochemical p53 overexpression and progression of pituitary adenomas^{34,35}.

1.2.1.1.2 Radiological parameters: invasion of the surrounding areas

While the classification of “atypical” pituitary adenomas mainly refers to histological markers, the term “aggressive” is not yet well defined for these types of tumors. Considering that an aspect of aggressiveness is the invasion into surrounding anatomical structures, the terms “aggressive” and “invasive” have been used interchangeably in literature. Great variation can also be found in the definition of pituitary invasiveness based on imaging features, histological proof of sphenoid sinus mucosal invasion and/ or intraoperative findings³⁶. Furthermore, the precise anatomical structure infiltrated is of importance since invasion into different areas is not always indicative of aggressive behavior³⁷. The application of Knosp’s and Hardy’s radiological classifications provides a useful tool for evaluating the extent and type of the invasion in pituitary tumors but is missing a correlation with the clinical course that could shed light on their aggressiveness^{38,39}. A possible solution for this problem could consist in the combination of both proofs of invasiveness and histological findings⁴⁰.

1.2.2 Clinical manifestations of pituitary tumors

Patients with pituitary tumors present a variety of signs and symptoms that can be generally divided into three categories:

- a) Signs and symptoms related to, or caused by, excessive hormone production

- b) Signs and symptoms related to mechanical effects of the expansion of the tumor within the *sella turcica*, which could include visual disturbances and headaches
- c) Signs and symptoms of impaired normal pituitary function (partial-or panhypopituitarism).

The following paragraphs will provide a brief description of the pathologic outcomes of pituitary adenomas, with particular attention to acromegaly.

1.2.2.1 Acromegaly and gigantism

The hallmark of acromegaly and gigantism is the presence of a chronic hypersecretion of GH and an excess of circulating IGF-1. Patients can be affected by acromegaly or gigantism depending on the age of onset of the disease: acromegaly has usually its manifestation in adults and gigantism in children. The etiology of the disease is usually a GH-secreting pituitary adenoma (>98%)⁴¹. Rarely, acromegaly may evolve due to hypothalamic tumor secreting GHRH or ectopic growth hormone releasing hormone secretion, or even more rarely due to GH from an ectopic source⁷. The incidence of acromegaly is about 3-4 cases per million, with a prevalence of 40-60 cases per million. No known sex, ethnic or racial differences are reported^{25,42}. The peak incidence of the disease is around the fourth decade of life but patients may present with acromegaly at all ages⁴³. Acromegaly is associated with a panel of different comorbidities. Although the over-secretion of GH can cause severe morbidities itself, most of the complications are due to the increased production of the GH-dependent factor IGF-1, synthesized in almost all body tissues, especially in the liver²⁵. The disease is generally characterized by disproportionate skeletal, tissue and organ growth. At diagnosis (that usually occurs

when clinically obvious later stages are manifested, 5-10 years after the first symptoms) patients may present a wide range of clinical features which include skeletal and peripheral overgrowth and soft tissue enlargement with frontal bossing, mandibular prognathism, jaw malocclusion and overbite, skin thickening, as well as increased ring and shoe size^{44,45}. Moreover, patients often present with several comorbidities including diabetes mellitus, headaches, visual symptoms, hypertension and other cardiovascular symptoms. Respiratory disorders can develop in acromegalic patients due to anatomical changes in the soft tissues and the craniofacial bone as well as alterations in the activity of respiratory muscles, which can lead to sleep apnea in up to 60% of cases⁴⁶. Arthropathy affects nearly 75% of patients with acromegaly and is the leading cause of functional disability in these patients⁴⁷. Examination of data regarding cancer incidence in acromegalic patients showed a higher risk of developing colorectal cancer⁴⁸. However, more recent studies from the German Acromegaly Registry did not show any indications for an association between acromegaly and the incidence of a particular type of cancer. All in all, the prolonged presence of some of the comorbidities leads to reduced life expectancy⁴⁹.

1.2.2.1.1 Medical management of Acromegaly

Analysis of the principal causes of mortality in patients indicates that ~60% of acromegalic patients die from cardiovascular diseases, 25% from respiratory diseases and 15% from malignancies. In view of all of the adverse influences of GH/IGF-1 excess, treatment of all patients is indicated. Transphenoidal adenectomy is usually considered as the treatment of choice. The main goals of the treatment are the reduction of GH values <2.5 µg/l and normalization of IGF-1 levels (adjusted for age and sex), preserving normal pituitary function and reversing the mechanical

effect of the tumor in the surrounding structures^{25,50}. The adenectomy, depending on tumor size and location, preoperative GH concentration and expertise of the surgeon, is usually successful in reaching these goals in 50% of the cases⁴². Patients with residual tumor post-operatively or who are not good candidates for surgery require additional treatments for normalizing excessive GH secretion: these include radiotherapy and medical treatment. Radiotherapy can be effective in controlling tumor growth in 70-80% of patients, but hypopituitarism could arise in the long term period. Therefore, this treatment is generally regarded as third line option behind surgery and medical therapy⁵¹. The latter, is usually chosen to treat persistent or recurrent acromegaly after non-curative neurosurgery. Medical therapy can be also used as a primary therapy for patients in whom surgery is contraindicated or as a short-term therapy before the intervention^{23,52-54}. Somatostatin Receptor Ligands (SRLs) were shown to effectively control GH excessive secretion in 50-70% of patients with partial amelioration of some of the co-morbidities⁵⁵⁻⁵⁷. Dopamine agonists were only effective in 10% of the patients and showed higher efficacy when combined with SRLs in presence of coexistent hyperprolactinemia, especially with long-acting treatments⁵⁸⁻⁶⁰. The GH-receptor antagonist pegvisomant was effective in normalizing IGF-1 levels in 67.5% of patients after 5 years of treatment. Due to the fact that it acts on target tissues for GH, it did not show to have an effect on tumor volume reduction^{7,52,61}. The implementation of an efficient medical treatment is currently under development with the design of new therapeutic options which could improve the control of GH-and IGF-1 levels, reducing the impact of comorbidities that arise with the progression of the disorder⁷.

1.2.2.2 Hyperprolactinemia

The symptoms of patients suffering from hyperprolactinemia may be due to the direct effects of excessive prolactin levels, the onset of galactorrhea or hypogonadism or the effects of the expanding mass of the pituitary tumor (headaches, visual defects). Affected women affected present with menstrual abnormalities, or regular cycles with infertility. Men present first present with symptoms related to the expansion of the pituitary tumor or adrenal/thyroid failure, although they may suffer from loss of libido or impotence for many years before the manifestation of the pituitary mass-related symptoms⁶².

1.2.2.3 Cushing's disease

Cushing's disease is characterized by high cortisol blood levels and loss of its circadian oscillation. Clinical symptoms usually vary depending on the duration and plasma levels of cortisol⁶³; they may include obesity (centripetal fat deposition), signs of protein wasting (thin skin, abdominal purple to red and wide cutaneous *striae*, easy bruising), bone wasting leading to osteoporosis and possible fractures, hypertension, gonadal dysfunction and hyperandrogenism, as well as mild to severe psychic disturbances (depression, anxiety)⁶⁴.

1.2.2.4 Thyrotropinomas

The adverse effects of TSH-secreting thyrotropinomas can be traced back to excessive levels of the hormone in the thyroid gland. Thyroid manifestations in patients with TSH-secreting adenomas include goiter, thyroid nodules and hyperthyroidism. Symptoms of mass effect like headaches and visual field defects also occur as most thyrotropinomas are macroadenomas at presentation⁶⁵.

1.2.2.5 Gonadotropinomas

Gonadotroph adenomas are difficult to diagnose because they are usually non-secreting, or they secrete biologically inactive peptides with no clinical effects.

Moreover, they usually grow silently until neurological symptoms develop. Clinical signs or symptoms of gonadotropin hypersecretion are very rarely reported, involving a few premenopausal women with ovarian hyperstimulation syndrome and men with macroorchidism⁶⁶.

1.2.2.6 NFPAs

The category of NFPAs comprehends all the pituitary adenomas that are not hormonally active. At the present time, NFPAs are diagnosed either in the context of mass effect related to the expansion of a macroadenoma or during imaging for some unrelated purpose; the latter case is known as pituitary incidentaloma⁶⁷.

1.3 Pituitary tumorigenesis

Pituitary tumorigenesis is a complex process in which intrinsic and extrinsic factors can participate. In fact, pituitary tumors may arise from alterations of pituitary cells or due to the dysregulation of regulatory factors like hypothalamic releasing and inhibitory hormones, peripheral hormones and paracrine growth factors. There are two main theories on pituitary tumorigenesis: the first implies a single mutational event in a single cell that will generate a monoclonal tumor, the second one asserts that the hypothalamus failing its regulatory role leads to a hyperplastic phenomenon in the target pituitary in which a mutational event provokes tumor formation. The monoclonal nature of the majority of pituitary tumors was elucidated by X-chromosomal inactivation analysis in female patients^{68,69}. This assumption was

confirmed by both loss of heterozygosity (LOH) analysis and X-chromosome inactivation, but it was also shown that recurrent tumors are frequently derived from independent clones of the initial tumor, indicating that there may be more than one clone, with one dominating the overall picture⁷⁰. On the other side, there are several animal models providing support for the hypothesis that pituitary tumors develop from a background of hyperplasia of the respective cell type. Moreover, the importance of hypothalamic factors and peripheral hormones in influencing tumor growth and progression was supported by clinical findings^{71,72}.

In addition to their origin, another important aspect to consider for understanding the biology underlying pituitary tumorigenesis is cellular growth. In fact, a distinctive feature of pituitary tumors is their slow expansive growth that is associated with a benign nature of the tumor in the majority of cases, rarely progressing to carcinogenesis. Considering that benign tumors usually present a senescent-like phenotype that is not present in their malignant counterparts⁷³, and that low pituitary tumor proliferative activity might be reflective of a proliferative arrest⁷⁴, cellular senescence has been hypothesized to be a novel mechanism that could drive pituitary tumors development⁷⁵, in a process that might involve alterations in gene expression. Thus, the following paragraph will provide an overall description of the genetic causations of pituitary tumors, with particular attention to pituitary senescence.

1.3.1 The genetics of pituitary adenomas

Pituitary tumors can occur because of the activation of proto-oncogenes or the inactivation of tumor-suppressor genes (TSGs), and by epigenetic mechanisms combining these two processes. Oncogenes are responsible for a gain of function and are dominant at cellular level, requiring a single "hit" in order to lead the cell to

tumorigenesis. Conversely, a TSG only loses its “anti-tumor protection” role only when both alleles are lost⁷⁶. Oncogenes (*RAS*, *MYC*) and TSGs (*p53*, *RB*) mutations commonly encountered in non-endocrine neoplasms are rarely present in pituitary adenomas, but do occur in particularly aggressive tumors^{77,78}. Several other genetic alterations have been implicated in pituitary tumorigenesis; that here are briefly listed and described here.

Heterozygous activating somatic point mutations in the α -subunit of the stimulatory Gs protein (*G α s*) were the earliest dominant activating mutations described in pituitary tumors. The activated oncogene stimulates proliferation and GH hypersecretion involving the activated form of the transcription factor cyclic AMP Response Element Binding Protein (*CREB*)^{79,80}. The mammalian securin, known as Pituitary Tumor-Transforming gene (*PTTG*) is a proto-oncogene required for pituitary tumorigenesis; it induces tumor transformation when overexpressed and its levels correlate with tumor angiogenesis as well as to tumor invasiveness and aggressiveness⁸¹. Several conditions are associated with a known genetic defect predisposing to pituitary adenomas. These include multiple endocrine neoplasia type 1 (*MEN1*) and Carney complex (*CNC*), which are related to mutations of the TSG *MEN1*, and protein kinase A regulatory subunit-1-alpha (*PRKAR1A*), respectively⁸². Truncating mutations in the cyclin-dependent kinase inhibitor 1B (*CDKN1B*) gene or in its upstream open reading frame (uORF) defined susceptibility for a novel type of endocrine neoplasia termed MEN4^{83–85}. Mutations in the aryl hydrocarbon receptor interacting protein (*AIP*) gene are associated with a predisposition to pituitary adenoma formation⁸⁶. McCune-Albright syndrome caused by activating mutations in the guanine nucleotide-activating alpha-subunit gene (*GNAS*), could lead to PA in a hereditary setting, but no familial cases have been

reported to date^{87,88}. Recently, the duplication and a recurrent mutation of the G-protein-coupled receptor 1 (*GPR101*) gene has been associated to the development of a new pituitary gigantism syndrome in young children called X-LAG acrogigantism (X-LAG)⁸⁹. In addition, somatic mutations affecting the deubiquitinase gene *USP8* have been reported in a high percentage of ACTH-secreting pituitary adenomas⁹⁰. Several other TSGs have been associated with pituitary tumorigenesis, but their abnormal expression was not reported in correlation with mutations: these include the zinc-finger protein pleomorphic adenoma gene-like 1 (*ZAC*)⁹¹, the Growth arrest and DNA damage-inducible protein gamma (*GADD45G*)⁹², and maternally expressed protein 3A (*MEG3A*)^{81,93}.

1.3.2 Pituitary senescence

Pituitary adenomas very rarely progress to become true metastatic carcinomas and exhibit stable growth even after decades of observation⁹⁴. Considering this usually low proliferation rate, senescence could be proposed as a candidate to explain the benign behavior of pituitary tumors. Cell cycle dysregulation was reported in pituitary adenomas, including overexpression of cyclins, and downregulation of cyclin-dependent kinases and Retinoblastoma Protein (Rb) expression. The altered expression of cytokines and growth factors was also observed in pituitary tumors. In particular, IL-6, which has an important role in pituitary tumor progression, has been found to have a pivotal role in induction and maintenance of oncogene-induced senescence (OIS), suggesting its role as stimulator of adenoma progression inducing OIS^{95,96}. The gene *PTTG* is correlated with invasiveness, recurrence, poor prognosis, and tumor metastasis^{97,98} and its overexpression is required for pituitary tumorigenesis^{99,100}. *PTTG* deletion results in pituitary-specific senescent features, including upregulation of the cyclin-dependent kinases inhibitors p15^{INK4B},

p16INK4A, and p21CIP1, and hypophosphorylation of Rb^{97,98,101}, overexpression of cyclin D1, apoptosis block, and elevated senescence-associated β -galactosidase (SA- β Gal) expression^{74,102,103}. A representative illustration of model for the induction of cellular senescence during the development of pituitary adenomas is shown in Figure 1.3.

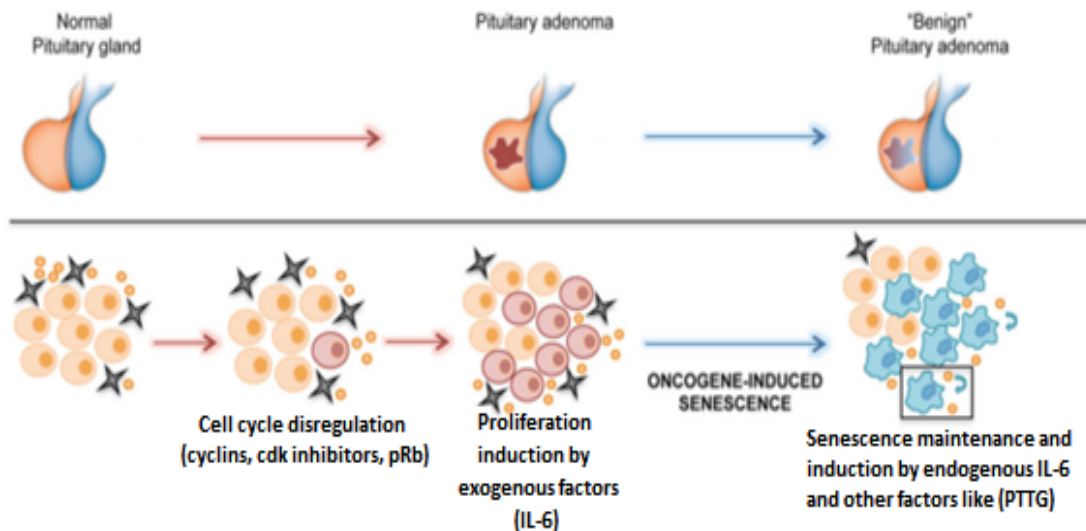


Figure 1.3: Representative model of the induction of senescence during pituitary tumor development. The initial proliferative phase of pituitary tumors is followed by a “stoppage phase” due to cellular mechanism activated by OIS, resulting in a benign tumor with stable growth arrest. Adapted from Sapochnik et al., 2015¹⁰³.

1.4 AHR-HSP90-AIP complex

The novel molecular mechanisms for pituitary tumorigenesis investigated in this thesis are linked to AIP protein, for which there is a clear evidence of an involvement in the pathogenesis of pituitary adenomas.

1.4.1 AHR

AHR is a member of the basic helix-loop-helix/ Per-ARNT-Sim (bHLH/PAS) family of transcription factors, formerly discovered as cytosolic receptor for the persistent environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD, also commonly referred to as TCDD or dioxin)^{104,105}. AHR was found after its activation ligand-mediated to up-regulate cytochrome P450 (CYP) CYP1A1 and CYP1A2 and other gene products, mainly involved in xenobiotic metabolism¹⁰⁶. Later on, a wide variety of structurally different compounds was found to bind AHR, including other aromatic environmental pollutants, pharmaceuticals, and compounds of natural origin synthesized by microbes, plants or endogenously within the human body¹⁰⁷. However, in addition to TCDD, the majority of high-affinity AHR agonists are synthetic chemicals, including polychlorinated biphenyls (PCBs) dibenzo-p-furans (PCDF) and dibenzo-p-dioxins (PCDD), benzo[a]pyrene (BaP), 3-methylcholanthrene (3MC) and β -naphthoflavone (β NF)^{107,108}.

1.4.1.1 AHR signaling

AHR possesses both nuclear localization (NLS) and nuclear export sequences (NES), therefore is continuously shuttled between the nucleus and the cytoplasm. In absence of ligand AHR is inactive, confined in the cytoplasm and associated in a complex formed by two heat shock protein 90 (HSP90), p23 and AIP molecules. AHR is also reported to interact in its inactive form with other cytosolic proteins including the chaperone Cdc37 and the non-receptor tyrosine kinase Src, exerting a crosstalk with the MAPK signaling cascade^{109,110} (Figure 1.4). The HSP90 molecules have the function to mask the ligand-binding site and the NLS in the AHR molecule¹¹¹, whereas AIP interacts with AHR and HSP90 in order to stabilize the

complex¹¹². Moreover, AIP seems to protect AHR against ubiquitination and proteosomal degradation¹¹³. The co-chaperone p23 plays a role in ligand responsiveness and the process of AHR activation¹¹⁴.

Ligand-binding induces morphological alterations in the structure of AHR allowing it to switch into its active form and dislocate from the stabilization complex. Exposure of the NLS leads to the translocation of AHR in the nucleus, where it dimerizes with its partner AHR nuclear translocator (ARNT), also known as hypoxia inducing factor β (HIF-1 β). The formed heterodimer is transcriptionally active and binds to xenobiotic responsive elements (XREs), which contain the core bases 5'GCGTC-3' in promoter enhancer regions of target genes. Large scale analyses have been conducted in order to understand the genes influenced by AHR activation, but the results obtained were differently variable in relation to different cellular models tested and the dependence on the chemical properties of the AHR ligands used. Despite the variability observed, results showed little overlap for a subset of target genes named after "The AHR battery". These genes include *CYP1A1*, *CYP1A2*, *CYP1B1*, *AHRR* (AHR repressor), *NQO1* (NAD(P)H quinone oxidoreductase 1), *ALDH3A1* (aldehyde dehydrogenase 3 family, member a1), *UGT1A6* (UDP glucuronosyltransferase 1 family, polypeptide A6), and *GST-Ya* (glutathione S-transferase)¹¹⁵.

In addition to the well-known effects of AHR activation in response to the ligand TCDD (wasting syndrome, teratogenicity, hepatotoxicity, neurotoxicity, immunotoxicity, and oncogenesis), several studies have elucidated the effects of AHR activation at the molecular level. For example, constitutive overexpression of AHR in transgenic mice reduced the life span of the mice and induced tumors in liver and in the glandular part of the stomach^{116,117}, suggesting the necessity for the

cell to regulate AHR levels by avoiding its excessive activation or expression in order to maintain cellular health. In fact, feedback mechanisms for regulating AHR activation were discovered at different steps of the molecular signaling cascade (Figure 1.4):

- a) Xenobiotic metabolizing enzymes oxidize polycyclic aromatic hydrocarbons, facilitating their removal by ATP dependent membrane transporters¹¹⁵;
- b) The cellular pool of AHR is reduced by proteasome degradation¹¹⁸;
- c) The activity of AHR is modulated by NES present in AHR¹¹⁹;
- d) AHR activation induces the expression of AHRR due to the presence in *AHRR* promoter of XRE sequences (see paragraph 1.4.2.4 and 1.4.2.5)¹²⁰.

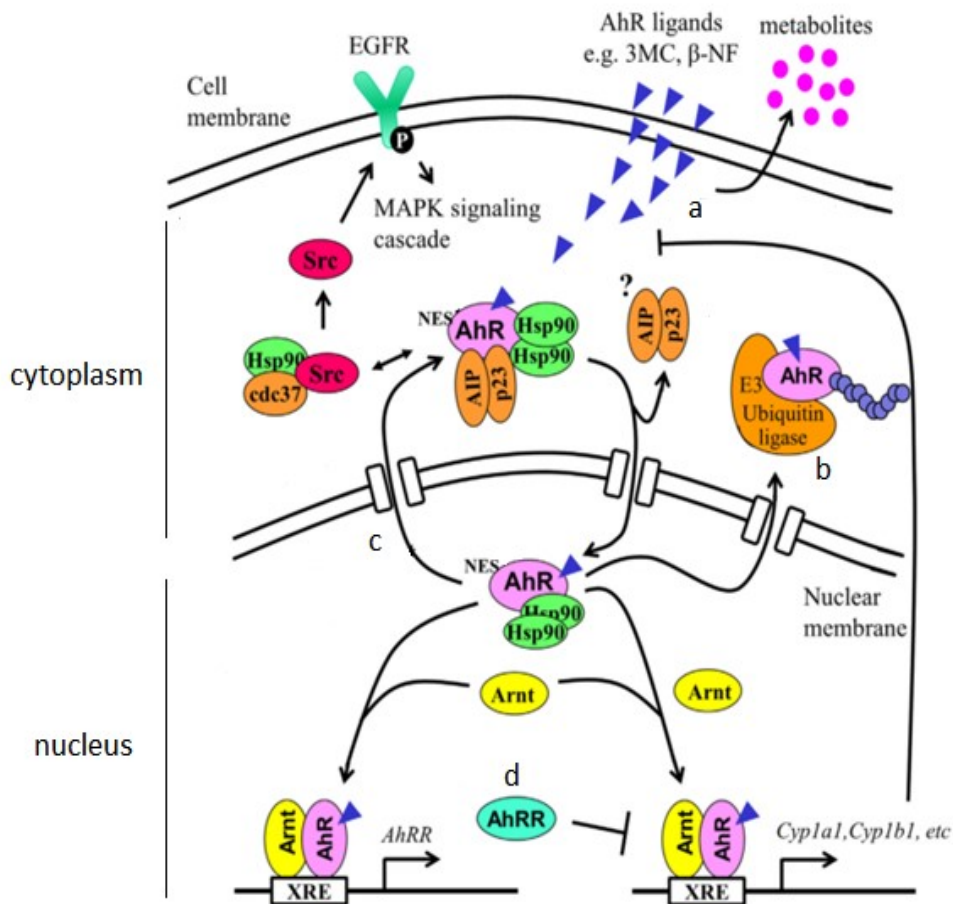


Figure 1.4: Schematic pathway representing AHR activation and regulation. In absence of ligand, AHR is mainly located in the cytoplasm, forming a complex with AIP, p23, HSP90, Cdc37 and Src. The binding of the ligand induces a conformational change in AHR switching it to its active state, resulting in nuclear translocation and release of the associated proteins. Activated AHR dimerizes then in the nucleus with ARNT and the neo-formed heterodimer induces the transcription of target genes including CYP1A1 and CYP1A2 involved in xenobiotics metabolism and AHRR. Different mechanisms act for regulating AHR activity or expression: a) xenobiotic metabolizing enzymes mediate ligand degradation; (b) AHR is degraded in the proteasome; (c) AHR is exported in the cytoplasm due to NES; (d) AHR/ARNT induces the expression of AHRR. Adapted from Hao et al., 2013¹¹⁵.

1.4.1.2 The tumorigenic role of AHR activation

Apart from the role of AHR in mediating the effects of exogenous ligands, its endogenous expression has been correlated with many biological processes including development, immunity and cancer biology^{121–123}. A recent study from a panel of 967 different cell lines from the Cancer Cell Line Encyclopedia reported that AHR is expressed among a wide variety of cell lines including those from breast, liver, lung, prostate, stomach and colorectal cancers. Skin-derived cell lines and liver cancer cell lines express relatively high levels, whereas many subtypes of leukemia cells express low AHR mRNA levels¹²⁴. Genetic and mutagenesis studies *in vitro* in cancer liver cell lines proved that AHR expression and activity have tumor-promoting effects, influencing growth potential and cell-cycle dysregulation¹²⁵. In contrast to these findings, experiments conducted by inducing liver adenomas in transgenic AHR^{-/-} mice suggested a tumor-suppressor like activity of AHR¹²⁶. The role of AHR has also been investigated in cell lines derived from other types of tumors, exhibiting both oncogenic and tumor-suppressive properties in a cell context-dependent way. AHR knockdown in H508 colon cancer cells did not affect cell growth; conversely, *in vivo* studies in transgenic AHR^{-/-} mice showed that the loss of AHR resulted in the spontaneous formation of colonic polyps and cecal tumors¹²⁷. Variable results were also obtained in breast cancer derived cell lines: AHR silencing in MCF-7 and BT474 cell lines and the B α P resistant cell line T47D (expressing low levels of AHR) showed increased cell proliferation, whereas slow growth was reported in B α P resistant MCF-7 cells^{128,129}. Immortalized mammary tumor fibroblasts derived from AHR^{+/+} and AHR^{-/-} mice were used as models for showing that AHR is required for tumor growth in a subcutaneous mouse xenograft model and AHR loss was associated with decreased migration and angiogenesis¹³⁰. Constitutive expression

of AHR in transgenic mouse models led to the development of stomach lesions, stomach cancer and liver cancer^{116,117}, whereas constitutive AHR overexpression in MCF-7 cells and Jurkat T cells inhibited growth in both cell lines and also induced apoptosis in Jurkat cells^{131,132}. These reports seem contradictory but they could just reflect differences in the impact of AHR activation that are cell-context dependent or could differ between adenomas and carcinomas. A novel description of the consequences of AHR activation and its relation to tumorigenesis could be found in cellular senescence. In fact, cellular senescence plays an important role in carcinogenesis as demonstrated in RAS-induced tumorigenesis model of premalignant and malignant lung adenomas, suggesting the role of senescence is a barrier that tumors have to overcome in order to reach their malignant form⁷³. In the study by Ray et al. 2003, primary human keratinocytes were cultured in presence or absence of the AHR ligand TCDD. The activation of AHR increased the differentiation of the cell line and led to the inhibition of SA- β Gal staining in the differentiating keratinocytes accompanied by decreases in cell cycle regulatory proteins p53, p16INK4a and p14ARF, supporting the idea that the activation of AHR by TCDD is a tumor-promoting event through the inhibition telomere-independent senescence response¹³³. These findings may be restricted to this peculiar cellular context; therefore, further studies need to be performed in order to clarify the hypothesis of senescence as a barrier for preventing malignant transformation¹³⁴.

1.4.1.3 AHR and the pituitary gland

The effect of chemical pollutants has been extensively studied in the endocrine system, due to the finding that dioxin-like chemicals could interfere with the endocrine system by affecting development and reproduction. For this reason

pollutants which have been discovered to work as agonist or antagonists to natural hormones have been generally named after endocrine disruptors (EDs). AHR is widely expressed in endocrine tissues and its activation by dioxin-like ligands has been shown to modulate pituitary function. The first evidence of the effect of AHR activation in the pituitary has been reported by Elango et al., 2006. In this study, exposure of rainbow trout pituitary cells to TCDD induced both GH and PRL mRNA expression partly through the modulation of the activity of AHR¹³⁵. An *in vitro* study by Moran et al., 2012 investigated the role of AHR activation in pituitary cells with a different AHR ligand. GH3 cells in which AHR was activated by treatment with β -naphthoflavone showed a suppression of AHR expression and an impairment of the expression of PRL without affecting the expression of GH mRNA¹³⁶. More recently, treatment of primary cultures obtained from mouse pituitary glands with dioxin-like PCBs displayed no effect in modulating apoptosis¹³⁷. Taking into account the long-term presence of TCDD in the human body, Landi et al., 2003 investigated the incidence of pituitary tumors in the population exposed to the pollutant following the industrial accident in Seveso area in 1976¹³⁸. Despite the impressive availability of epidemiological and clinical data, no increase of the incidence of pituitary adenoma was registered in the involved area¹³⁹. In one study evaluating the expression of AHR in normal pituitaries and in a subset of human pituitary adenomas, a contemporary reduction in AIP and AHR expression was detected in the most invasive tumors independently from the presence of AIP mutations, suggesting a possible correlation between AHR and tumor aggressiveness¹⁴⁰. Additional findings confirmed the latter hypothesis. The AHR gene *rs2066853* polymorphism was found to be more frequent in acromegalic patients than in healthy patients, and was also associated with increased disease aggressiveness¹⁴¹. More recently, genetic

variants potentially affecting AHR pathway were found to be associated with a more severe acromegaly, increased pituitary tumor size, and somatostatin analog resistance in patients living in highly polluted areas¹⁴².

1.4.2 AHRR

AHRR is a member of the bHLH/Per-ARNT-Sim protein family and has been found to act as a repressor of the AHR function¹⁴³. It has been formerly isolated by Mimura et al., 1999 during the screening of a mice genomic library using AHR as hybridization probe. The newly discovered polypeptide was able to repress the transcription activity of AHR by competing with AHR in forming a heterodimer with ARNT and binding to XREs, also called AHR responsive elements (AHREs). The expression of AHRR was induced by the AHR/ARNT heterodimer through binding to the enhancer sequence XRE upstream of the AHRR gene, establishing a feedback negative mechanism for the regulation of AHR activity¹²⁰. However, further studies determined that the mechanism of AHR repression seemed to be more complex than the one reported herein (see paragraph 1.4.2.5). AHRR shares high amino acid (aa) identity with AHR for ~275aa in the N-terminal part of the protein that contains the bHLH and Per-ARNT-SIM domains, but the two proteins are highly divergent thereafter¹⁴⁴. Consistent with the lack of the PAS-B domain that in AHR is responsible for ligand-binding, AHRR is reported to act in a ligand-independent manner¹²⁰.

AHRR is constitutively expressed at mRNA levels in many adult tissues, especially in testis, lung, spleen, heart and kidney. In some tissues that express high mRNA AHR levels, such as in the liver, basal AHRR expression is low and in some cases it was just detectable after the induction of AHR activity with its ligands, thus confirming the interdependence between AHRR and AHR^{143,145–148}. However, it is

important to consider that mRNA expression levels did not always correlate with the expression at protein level. Hence, further data is needed in order to understand the more about the regulation of AHRR expression¹⁴⁴. In addition to the presence of the XRE enhancer sequence, several other regulatory elements were identified in the upstream region of the AHRR gene. These include GC box sequences that are responsible for AHR-ligand induced AHRR transcription in murine or human models¹⁴⁹, and an NF- κ B binding site which promotes AHRR transcription after the stimulation with an NF- κ B activator¹²⁰. These regulatory elements are also supposed to regulate AHR levels in basal conditions¹⁴⁴.

Finally, AHRR was also suggested to play a role in development, following the finding of its high expression in fish and amphibian embryos and in mammalian fetuses compared to adult tissues^{144,150–154}. Some studies had contradictory results in supporting this theory: in fact, knockdown of one of the two AHRR paralogs that have been found in zebrafish caused developmental abnormalities, whereas transgenic mice lacking AHRR did not show abnormal development^{155,156}, underlining that further investigations are needed in order to clarify this putative role of AHRR.

1.4.2.1 Hypotheses for AHR repression

There is no doubt that the primary role of AHRR is repressing the activity of AHR. Several studies have extensively demonstrated this function, mainly through investigating the expression of the genes prominently implicated in the AHR pathway (AHR itself, CYP1A1) in relation to the basal levels of AHRR in different tissues or after the modulation of the expression of one of these genes in different cell lines. However, the molecular mechanisms driven by AHRR have not been

completely explained. It is clear by now that AHRR is ligand-independent (due to the absence of the PAS-B domain, which is part of the ligand binding pocket is AHR)¹⁴⁴ and constitutively localized in the nucleus (the NLS of AHRR is dominant over its NES)¹⁵⁷, but the molecular mechanism by which AHRR represses the transcription of AHR is still uncertain. The authors of the initial studies in which AHRR was defined as the repressor of AHR proposed two mechanisms of repression: the competition between AHR and AHRR for binding with ARNT and the competition of the heterodimers AHR/ARNT and AHR/AHRR for binding to the DNA. The demonstration of the effective binding of AHRR to ARNT and the binding of the complex to AHREs made both of these hypothesis seem plausible¹²⁰. A further analysis ended up with a more complex molecular mechanism explaining the interactions between AHR, AHRR and ARNT. The overexpression of ARNT showed no effect in the AHRR-mediated repression of AHR, demonstrating that the competition between AHR and AHRR for ARNT is not the primary mechanism that occurs in AHR repression. In addition, a mutant defective in AHRE binding but with an intact NLS was constructed to test the competition for binding to AHRE, demonstrating that even the binding to AHRE did not seem to be necessary for AHR repression, although a slight decrease in repressive potency of the mutant was reported. These results suggested an additional mechanism to the ones initially reported that might involve transrepression through protein-protein interactions, independent from binding to AHREs, but still the specific interactions involved need to be verified^{144,146} (Figure 1.5).

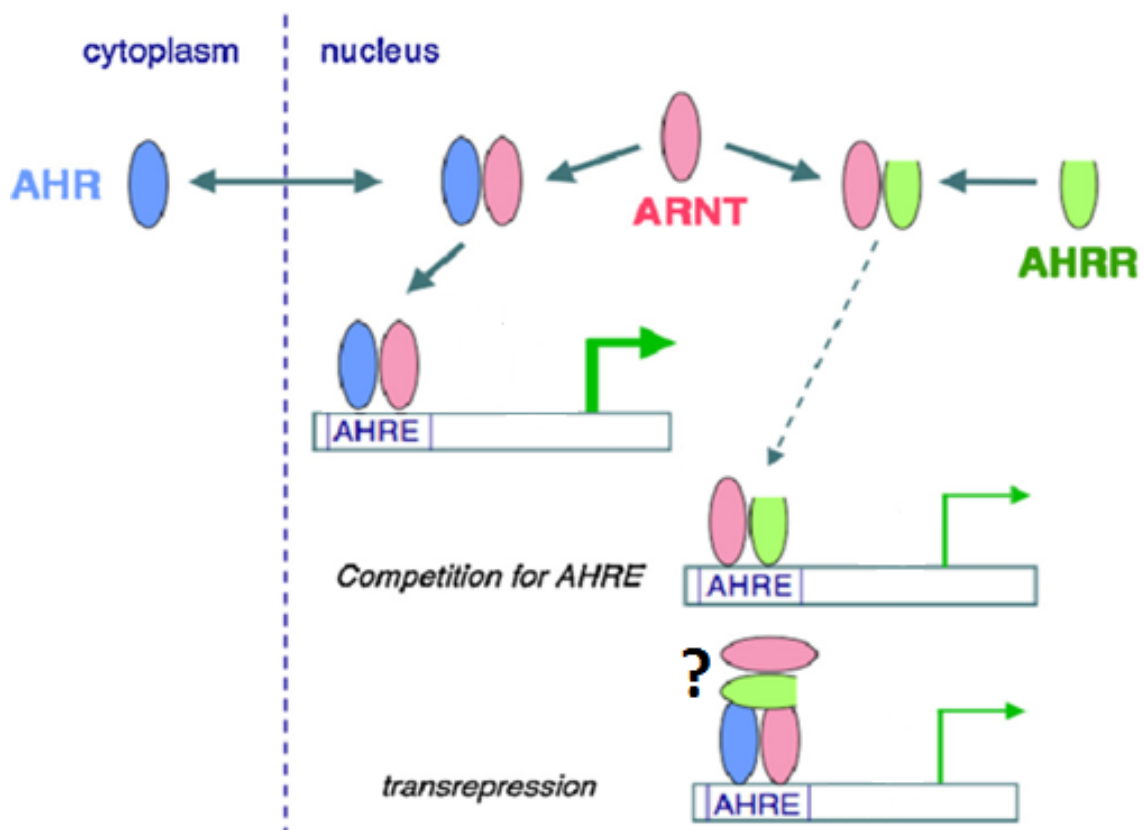


Figure 1.5: Mechanism of repression of AHR by AHRR. AHRR competes with AHR for dimerization with ARNT; moreover, AHRR/ARNT competes with AHR/ARNT for binding with AHREs, as proposed by Mimura et al., 1999¹²⁰. A second putative mechanism may involve transrepression, independently for competition with ARNT or AHREs, and it is these protein-protein interactions are ARNT-mediated or not. Adapted from Hahn et al., 2009¹⁴⁴.

1.4.2.2 AHRR and the pituitary gland

Due to the intensive investigation of dioxin-like pollutants as EDs, several studies have analyzed the impact of AHR activity in the epidemiology of pituitary tumors, but little is known about the role of AHRR. Two different studies investigated the response of AHR-responsive genes using *in vitro* and *in vivo* models, and both of them confirmed the presence of a functional response to the induction of AHR activity by some of its ligands in the pituitary. In the first study, an increase in the

mRNA levels of AHRR was reported after the treatment of AtT-20 cells with TCDD and β -NF, whereas only TCDD treatment was shown to induce AHRR mRNA expression *in vivo* in mice¹⁵⁸. In the second study, the response to TCDD exposure was investigated *in vivo* with the main aim to identify a possible connection between toxic damage by TCDD and vitamin A homeostasis. Despite the higher basal levels of AHRR in the pituitary of the knockout mice in comparison to the control strain, no difference was found in AHRR expression after AHR activation between knockout and wildtype mice¹⁵⁹.

1.4.2.3 AHRR and cancer

Several studies reported the involvement of AHR in the development of different types of tumors^{116,117,123}, but still little is known about the tumorigenic potential of AHRR. Kanno et al., 2006 demonstrated that AHRR overexpression in the human breast cancer cell line MCF-7 led to decreased cell proliferation and to decreased expression of cell-cycle related and estrogen-responsive genes^{160,161}. In addition, AHRR was found to be downregulated by hypermethylation in human malignant tissues from different anatomical origins including colon, breast, lung, stomach, cervix and ovary¹⁶². In particular, levels of promoter hypermethylation were closely correlated with cancer grade in cervical and esophageal cancer. These data suggest that in those tissues AHRR might act as a tumor suppressor gene. Furthermore, AHRR *in vitro* silencing in MCF-7 and A549 cell lines increased cellular growth and clonogenic potential in addition to motility, resistance to apoptosis and angiogenic ability¹⁶². At present AHRR expression in the pituitary has been investigated only in few studies^{147,158,159,163}. These studies underline the importance of further research

activity focusing on understanding the relevance of AHRR activity in tumorigenesis beyond the already known activity of AHR repression.

1.4.3 HSP90

HSP90 is an evolutionarily conserved and ubiquitous molecular chaperone required for correct folding and maturation of a variety of cellular proteins, accounting for 1-2 % of total cellular protein levels even under basal conditions^{164,165}. It has been detected in almost every compartment of eukaryotic cells, including cytosol, nucleoplasm, endoplasmic reticulum and mitochondria. In the eukaryotic cell, HSP90 exerts essential housekeeping functions by stabilizing or activating a vast array of proteins, generally termed as 'clients', which comprehend more than 200 proteins by now (for an up-to-date list refer to <https://www.picard.ch/downloads>). These encompass kinases, transcription factors and an array of signaling molecules involved in a wide range of biological processes¹⁶⁵⁻¹⁶⁷. HSP90 can also promote protein degradation through a passive activity leaving clients unfolded after many rounds of chaperone binding and release, after which the protein is more likely to encounter the ubiquitin ligases that would mark it to proteasome degradation, as reported for Von Hippel–Lindau disease tumor suppressor (VHL)¹⁶⁸. HSP90 does not just exert its role in basal conditions, but especially in adaptive response to different sources of cellular stress (including heat, heavy metals, hypoxia and acidosis), in which its expression approximately doubles¹⁶⁴. Inducible transcription of HSP90 is controlled by the transcription factor Heat Shock Factor 1 (HSF1), which induces several genes in response to environmental stress¹⁶⁹. HSP90 plays also a role in cancer, due to the fact that cancer cells use the chaperone activity of HSP90 and its related proteins to protect mutated or overexpressed oncoproteins from misfolding and degradation¹⁷⁰. Although in general relatively abundant, HSP90 does

not function alone, but as a component of a larger molecular complex that includes other chaperones such as HSP70, co-chaperones, modulators of its activity and diverse accessory proteins.

HSP90 is a member of the GHKL superfamily (that also comprises DNA gyrase, histidine kinase and the DNA mismatch repair protein MutL)¹⁷¹. The protein is composed of a homodimer, and each monomer has a highly-conserved amino-terminal domain (NTD) connected to a middle domain, followed by a carboxy-terminal domain (CTD). NTD has a α/β sandwich structure in which the helices form a pocket. HSP90 binds ATP in NTD, hydrolyzing it and following the interaction with clients, and the stabilizing interaction between the two monomers seems to enhance this activity^{164,171,172}; this function was demonstrated as necessary for the biological function of HSP90^{173,174}. A middle segment connects the NTD and the CTD and has a role in ATP hydrolysis due to the presence of a key-catalytic residue¹⁷¹. The CTD of HSP90 is a dimer of a small mixed α/β domain that mediates the dimerization of the homodimer and is less conserved in sequence than the rest of the protein. Although a low-affinity nucleotide-binding site has not been confirmed, the five C-terminal residues forming the Met-Glu-Glu-Val-Asp (MEEVD) motif make up a highly conserved TPR domain-binding site, which mediates interaction with many co-chaperones¹⁷⁵. This binding site has a particularly important role, which is to facilitate the cooperative action of HSP40, HSP70 and HSP90 on client proteins to achieve client maturation¹⁶⁴.

1.4.3.1 HSP90 chaperone complex and cancer

HSP90 is involved in the maturation and stabilization of a wide variety of oncogenic proteins (Bcr-Abl, HER-2, EGFR, C-Raf, B-Raf, Akt, Met, VEGFR, FLT3, AR and

ER, HIF-1 α)¹⁷⁶, which has stimulated the investigation of its possible role in tumor progression. Increased expression of HSP90 or other HSPs has been mainly reported in human cancers, solid tumors and hematological malignancies¹⁷⁰. Recently, overexpression of HSP90 has also been reported in corticotroph adenomas¹⁷⁷. The first evidence of HSP90 altered expression in cancer was reported in one study in which HSP90 overexpression was found in humane acute leukemia cells and was correlated with increased proliferation¹⁷⁸. Later on, overexpression of HSP90 and HSP70 was reported in breast cancer and associated with bad prognosis, suggesting that HSP90 might act at different level in tumor development¹⁷⁹. An increased overexpression of HSP90 might reflect at physiological level the possibility for the tumor cell to survive in a condition of stress, and at molecular level to permit to the oncogenic client to exert its tumorigenic effect. Increased survival might be also linked to a resistance to apoptotic stimuli¹⁷⁰. For instance, some co-chaperones of HSP90 modulate tumor cell apoptosis, having mainly effects on Akt¹⁸⁰, tumor-necrosis factor (TNF)-receptors¹⁸¹ and NF- κ B function¹⁸². Oncogenic mutations of clients may probably lead to higher requirements for HSP90 function, probably due to an increased conformational instability of the mutated protein. One example of this phenomenon is provided by the tumor suppressor protein p53, which is encoded by the gene *TP53*. Several mutations in this gene lead to the protein with altered conformation and activity of the protein. Wildtype p53 interacts with HSP90 and is quickly marked to proteasome degradation^{183,184}; conversely, altered p53 proteins are longer retained by HSP90 and accumulate in the cell exerting their tumorigenic role. These data suggest that cancer progression can be highly dependent on HSP90 and its client activity.

Therefore HSP90 is being investigated as a potential target for cancer therapeutics¹⁸⁵.

1.4.3.2 HSP90 inhibitors

There are many reasons why HSP90 should be considered as a target for cancer therapy. First of all, as cited in the previous paragraph, HSP90 acts as a chaperone for diverse proteins which play a role in carcinogenesis. Secondly, as a consequence of the need for stabilization of mutated oncoproteins, HSP90 shows to have a higher affinity for ATP binding, which could favor the binding of inhibitors that interact in this region of the protein. Thirdly, HSP90 is more expressed in tumor cells when compared to non-tumorigenic cells, suggesting a vital role of this protein in tumor cell survival^{176,186}. Several HSP90 inhibitors have been discovered and designed so far, and can be generally divided based on their site of action, that is a) N-terminal and b) C-terminal domain of the protein.

a) N-terminal inhibitors

The research on HSP90 inhibitors started with the finding that ansamycin antibiotics bind the conserved nucleotide-binding pocket of HSP90, blocking the natural substrate for ATP binding¹⁸⁷. Structural and biochemical studies demonstrated that geldanamycin (GA) specifically inhibits only HSP90 by restraining it in its ADP-bound conformation and prevents the subsequent “clamping” of HSP90 around a client protein^{188,189}. In spite of the specificity of the compound, research for more efficient derivatives was indicated due to the high hepatotoxicity observed in animal studies¹⁹⁰. The testing of 17-AAG showed an impressive downregulation of multiple HSP90 protein clients and other downstream effectors, such as IGF-IR, Akt, iKK- α , iKK- β , FOXO1, Erk1/2 and c-Met, resulting in inactivation of NF- κ B, reduced cell

proliferation and decline of cell motility¹⁹¹. 17-AAG entered phase I trials and signs of therapeutic activity have been found in melanoma, breast cancer, prostate cancer, and multiple myeloma^{192–195}, while the few phase II studies performed did not show complete or partial response^{196,197}. One important issue about 17-AAG is that it leads to the activation of a heat shock response with the activation of the transcription factor HSF-1^{198–200}. Another ansamycin derivative, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), showed downregulation of B-Raf, decreased cell proliferation, reduced Mek/Erk signaling activation and induced the inhibition of telomerase activity in melanoma cells^{201,202}. Seven clinical trials with 17-DMAG (5 phase I, 2 phase II) have been reported in clinicaltrials.gov database until now^{176,203–205}. In addition to 17-AAG and 17-MAG, several different derivatives of GA and others distinct from GA have also entered clinical trials and are currently in the preclinical stage of development¹⁷⁶. Another important inhibitor of the N-terminal domain of HSP90 is radicicol, a macrocyclic natural antibiotic initially isolated from the fungus *Monocillium nordinii* and *Monosporium bonorden*^{206,207}. Radicicol activity in inhibiting HSP90 is comparable to that of ansamycin derivatives²⁰⁸. Despite from its antitumoral activity *in vitro*, the compound showed little or no activity *in vivo*, due to its chemical and metabolic instability^{209,210}.

b) C-terminal inhibitors

Generally unsatisfactory results with N-terminal inhibitors in experimental trials resulting from increased toxicity and stress response led to the focus on the C-terminal region of HSP90 for the development of novel and improved inhibitors. Novobiocin, a coumarin antibiotic isolated from *Streptomyces* species²¹¹, and its analogues clorobiocin and coumerymicin A1 were found to bind strongly to DNA

topoisomerase II but weakly to the HSP90 C-terminal binding site, inducing the degradation of client proteins such as v-Src, Raf-1, Erb2 and mutant p53^{212,213}. Moreover, novobiocin was also able to disrupt the interaction of the co-chaperones p23 and HSP70 with the HSP90 complex²¹⁴. Despite initially promising results, novobiocin showed low efficacy against cancer cells in further trials, leading to its disqualification as an HSP90 inhibitor in medical applications²¹⁵. Structural modifications of this compound led to the conception of analogues with 1,000-fold greater efficacy in antiproliferative assays against various cancer cell lines¹⁷⁶. For instance, the novobiocin-derived HSP90 inhibitor KU135 induced antiproliferative effects in Jurkat cells which were higher than the ones produced by 17-AAG, and caused the degradation of HSP90 client proteins. The compound was as potent inducer of the intrinsic apoptosis, and did not induce upregulation of HSP90 and HSP70 as 17-AAG did. Moreover, KU135 showed a G2/M arrest, whereas cells treated with 17-AAG accumulated in G1 phase in the cellular model used. These results suggest that KU135 is effective by potentially regulating signaling pathways different from those affected by 17-AAG treatment²¹⁶. A further investigation of the effects of KU135 in melanoma cells, in addition to confirming some of the findings of the previous study, reported that KU135 induced apoptosis and reduced HSP90 client proteins BRAF, Raf-1, cyclin B and cdc25²¹⁷. Tests on the more recent C-terminal inhibitor KU174 in prostate cancer cells exhibited robust antiproliferative and cytotoxic activity together with client proteins degradation and disruption of HSP90 native complexes without inducing a heat shock response in cells. In addition, in a pilot *in vivo* study in a rat xenograft tumor model displayed that KU174 was effective in reducing tumor volume without signs of apparent toxicity, although the presence of vehicle toxicity was reported, especially in the kidney²¹⁸. Silibinin,

the major bioactive constituent present in the flavonoid extract of milk thistle silymarin, has been widely used as a hepatoprotective agent as well as a nutritional supplement to protect the liver from diseases associated with alcohol consumption and exposure to chemical and environmental toxins²¹⁹. Recent studies have demonstrated that silibinin exerts cytotoxic activity against cancer cell lines, in this case by exhibiting HSP90 inhibitory activity²²⁰. The compound displayed effects on cell viability, induced G1 cell cycle arrest, inhibited EGFR and NF-κB, down-regulated survivin and suppressed angiogenesis^{220,221}.

Due to the finding of HSP90 overexpression in a series of corticotroph adenomas in comparison to normal pituitary, and considering the known interaction between the glucocorticoid (GR) receptor with HSP90²²², the effect of N-and C-terminal HSP90 inhibitors has also been evaluated in this type of pituitary tumors. In this case, silibinin caused the release of mature GR from HSP90, increasing the number of stable GR receptors and partially restoring glucocorticoid sensitivity¹⁷⁷. This discovery offers a novel therapeutic approach for the management of corticotroph adenomas, opening the door to HSP90 inhibitors as alternative agents in pituitary tumor treatment.

2. Aims of the study

The overall aim of the present study was to investigate novel molecular mechanisms involved in pituitary adenomas development, especially in the context of the GH-secreting ones.

The main objectives were:

1) To evaluate the impact of the modulation of AHRR in the development of pituitary adenomas. This study was performed through the establishment of stable transfectants from the somato-lactotroph cell line GH3 for AHRR silencing or overexpression; in addition, the expression of AHRR was evaluated in the pituitary in a cohort of patients with acromegaly and NFPAs and results were correlated with radiological and pathological parameters of pituitary tumor aggressiveness;

2) To investigate the role of HSP90 in the pathogenesis of GH-secreting adenomas and its possible consideration as a novel therapeutical target by testing different types of inhibitors of the activity of the protein.

3. Materials and methods

3.1 Pituitary adenoma patients (Study I)

Diagnosis and management of pituitary disease was done by physicians referring to international criteria^{223,224}. Diagnosis of a GH-secreting adenoma was established by typical clinical symptoms in conjunction with non-suppression of GH levels after standard OGTT administration, IGF-1 values higher than age-gender match, and RMN evidency of pituitary adenoma²²⁵. Prolactinoma has been diagnosed after manifestation of hypogonadism, high-molecular-weight prolactin-rhythm evaluation and RMN evidency of pituitary adenoma²²⁶. For the diagnosis of NS pituitary adenomas the absence of clinical and biochemical effects of the tumor is necessary⁶⁷.

3.1.1 Sporadic patients (Study I)

The selected cohort consisted of 51 patients that did not report cases of other pituitary adenomas in their families. 35 of these were GH-secreting-, 5 GH-and PRL-secreting and 11 were NS adenomas. Patients were recruited until April 2015 in the Endocrinology Division of Padua Hospital/University and operated at the Neurosurgery Unit of University/ Hospital of Padua. Tumor phenotype was confirmed through immunohistochemical characterization of hormone secretion and tumor aggressiveness was classified through radiological and histological criteria²²⁷. Pituitary tumor samples were maintained in RNA later stabilization solution (Thermo Scientific, Waltham, MA, USA) overnight at 4°C and subsequently stored at -20°C till the moment of RNA extraction.

3.2 Cell culture and treatments (Studies I, II)

The rat somato-lactotroph pituitary tumor cell line GH3 (American Type Culture Collection, Manassas, VA) has been used as a model in study I for evaluating the impact of the modulation of AHRR expression and in study II for testing the efficacy of HSP90 inhibitors. Cells were seeded in DMEM 10% prepared as follows: DMEM low glucose medium (Euroclone, Pero, Italy) supplemented with 10% FBS, 2mM L-Glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, Carlsbad, CA, USA) and the cells were propagated in a fully humidified atmosphere of 5% CO₂/95% air at 37°C.

For GH3 treatment in the study II the following compounds have been tested: 17-AAG (Tocris Bioscience Bristol, UK), novobiocin (Sigma-Aldrich, MI, USA) and KU174 (University of Kansas, KS, US.). All the tested substances were dissolved in DMSO. Cell treatments were performed 24 h after cell seeding by diluting the compounds or vehicle in DMEM 2% (DMEM low glucose supplemented with 2% FBS, 2mM L-Glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin).

3.3 Establishment of stable cell lines (Study I)

The stable expression of a gene of interest consists in its permanent expression in the cell line used. At first, the gene of interest has to be introduced into the cell, then subsequently into the nucleus, and finally has to be integrated into the chromosomal DNA (Fig. 3.1). The event of integration in the total population of cells transfected is rare and the only way to maintain the population stable is a sustained selection against those cells that have lost the plasmid. For the selection of stably-transfected cells, a selection marker is co-expressed on either the same construct or on a second, co-transfected vector. There is a variety of systems for selecting transfected

cells, including resistance to antibiotics such as neomycin phosphotransferase, conferring resistance to G418, dihydrofolate reductase (DHFR), or glutamine synthetase²²⁸. After transfection, cells are kept in medium containing the selective agent, and only those cells containing the drug-resistant gene who will have permanently integrated the plasmid will survive.

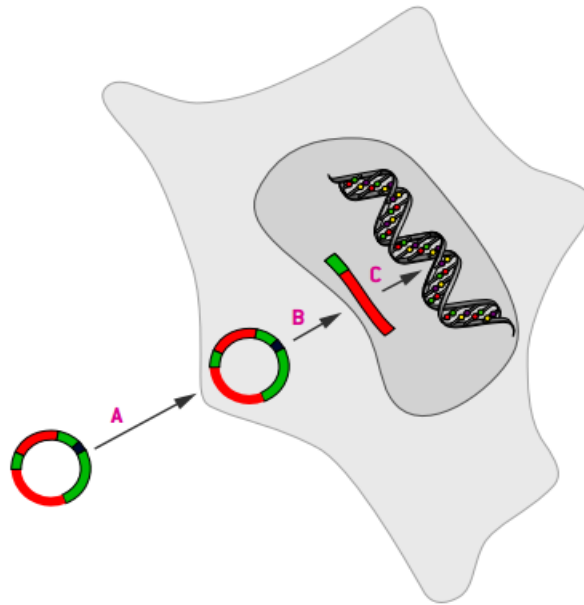


Fig. 3.1: General procedure for generating stable cell lines. The plasmid containing the gene of interest and the gene for drug selection are introduced in the cell through transfection (A). Subsequently, through prolonged drug selection the plasmid is integrated in the nucleus (B) and finally in the chromosomal DNA (C). Taken from <http://bio.lonza.com>

There are different choices for generating stable cell lines, depending on time and aim of the study. A mixed population for drug resistant cells can be used for experiments, having the possibility to obtain results in a relatively low amount of time, with the disadvantage of having a genetically mixed population because the plasmid will be integrated in random parts of the host chromosomal DNA. Another possible approach consists in diluting transfected cells on a large scale, so that each culture will theoretically be derived from a single clonal cell. An important

disadvantage of this technique is the amount of cell passages needed to reach a suitable amount of cells for testing and creating a storage, negatively influencing the reproducibility of the results due to an increased probability of cell differentiation. The generation of stable cell lines is useful for a wide range of applications, such as gene function studies, drug discovery assays, or the production of recombinant proteins.

In this work, stable cell lines were generated from the cell line GH3 for overexpressing or silencing of AHRR, establishing mixed populations as described in Figure 3.2. Twenty-four hours before the transfection, 7×10^4 cells/well were seeded in 24-well plates. Transfection was performed using 1 μ l of Lipofectamine 2000 (Invitrogen, Milan, Italy) and 0.8 μ g of plasmid DNA. Plasmids carrying shRNA sequences for AHRR silencing, overexpression and respective controls were generated by SABiosciences-Qiagen (Venio, Netherlands) (see appendix 1). To obtain stable cell lines, selection pressure was maintained by supplementing culture medium with puromycin (1 μ g/ml, Gibco) for a period of two weeks. After this period, puromycin concentration was scaled down to 0,5 μ g/ml and kept throughout the experimental period. Aliquots of cells have been frozen and kept in -80°C in order to prevent high number of passages and restored when necessary.

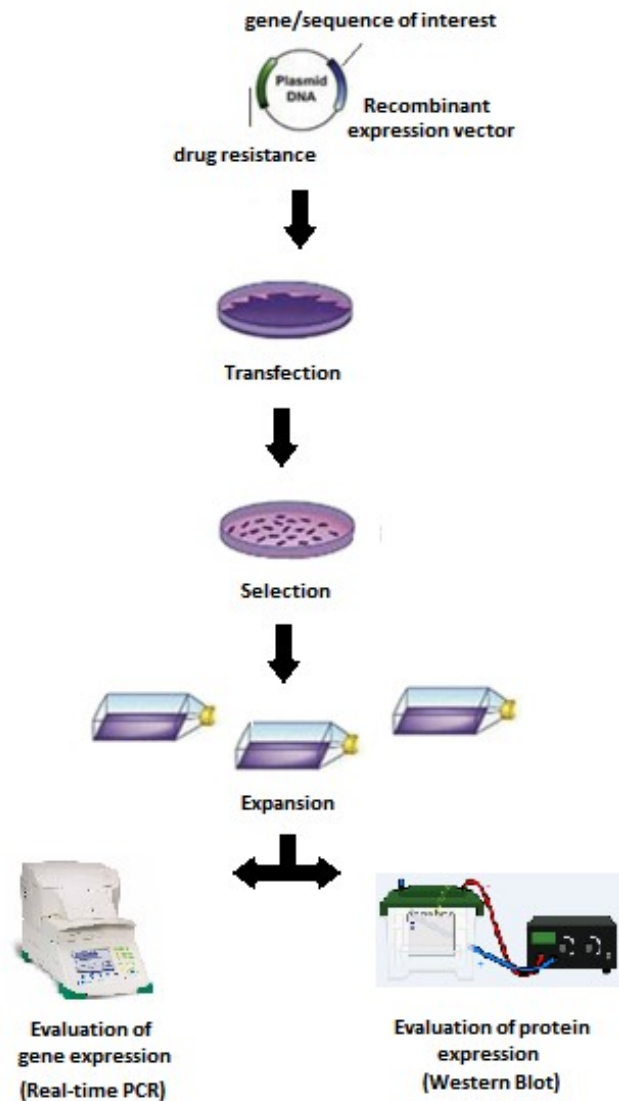


Figure 3.2: General approach for establishing mixed population of stable clones. Cells are seeded and transfected with the plasmid containing the gene of interest and the gene for the drug resistance. After the transfection, the selection medium is added and surviving cells are the ones who integrated the plasmid. Subsequently recombinant cells are expanded and tested for the expression of the gene of interest and the relative protein of interest (Modified from <http://www.creative-biogene.com>).

3.4 RNA Extraction (Study I)

Total RNA was extracted from GH3 cells to confirm at mRNA level the effective silencing or overexpression of AHRR and from pituitary tissues for evaluating AHRR mRNA expression in the cohort. Rat pituitary cells and human pituitary adenoma samples were lysed and homogenized in TRIzol (Invitrogen) and processed according to manufacturer's instructions. Briefly, chloroform (Carlo Erba, Val de Reuil, France) was added to the sample to separate RNA from proteins and DNA from RNA. The phase containing the RNA was collected and precipitated by adding isopropanol (Carlo Erba), and contaminants were washed away. The pellet containing RNA was then resuspended and washed with ethanol 75% (Carlo Erba) and when dried was furtherly resuspended in RNase free water.

3.5 Determination of RNA concentration and purity (Study I)

The yield of purified RNA was then determined by a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Using fiber optic technology and surface tension, this technique permits a high accuracy in determination of nucleic acids quality and concentration in microvolumes of samples. The concentration of the nucleic acids is automatically calculated by the NanoDrop software through the formula: $A_{260} \times 44 \mu\text{g/ml} \times \text{dilution factor}$. Purity of the DNA or RNA is determined by the ratio A_{260}/A_{280} . For RNA, a ratio between 1.8-2.0 indicates little or no protein contamination.

3.6 DNase treatment and determination of RNA integrity (Study I)

Total RNA was incubated with DNase I (RNase free) endonuclease (Thermo Scientific) according to the manufacturer's instructions, in order to remove genomic

DNA contaminations that could have derived from a non-excellent phase separation of the lysate with chloroform (see paragraph 3.4). To verify the integrity of the purified RNA and the quality of the purification, 500ng of the samples were run in a denaturing 1% agarose gel stained with Etidium Bromide solution (Sigma-Aldrich). The samples showed as expected 28S and 18S bands of ribosomal RNA (rRNA), with 28S bands approximately twice intense as the 18S band as good indication of RNA integrity. The absence prominent bands above the rRNA ones after the DNase treatment certified the removal of genomic DNA contamination.

3.7 Reverse Transcription of RNA (Study I)

Single strand cDNA was obtained in a reaction of reverse transcription by loading 500 ng of DNase-treated RNA and using random examers as primers ~~and~~ and M-Mulv reverse transcriptase (Euroclone, Pero, Italy), following manufacturer's instructions.

3.8 Real-time PCR (qRT-PCR) (Study I)

The technique of Real-time PCR (qPCR) is based on a polymerase chain reaction. The cDNA is amplified and the amount of nucleic acid is quantified through fluorescent reporter dyes. The increase in fluorescence signal during the reaction is proportional to the amount of DNA produced during each PCR cycle. Using probes with different reporter dyes allows the detection of multiple target genes in the same reaction. The parameter considered for determination of cDNA amplification is the quantification cycle (Cq), that is the number of cycles in which fluorescence exceeds the threshold. The more target is in the starting material, the lower is the Cq. Gene expression was quantified by qPCR with with an ABI PRISM 7900HT Sequence Detector (Applied Biosystems, Waltham, MA, USA) based on EXPRESS qPCR

Supernix containing a ROX passive reference dye and Taqman gene expression assays (Thermo Scientific) with coverage of all human (Hs01005075_m1) or rat (Rn01537444_m1) AHRR transcript variants. To compensate relative differences between samples the results obtained were corrected in rat pituitary cells for ACTB (Rn00667869_m1) and in human pituitaries for HMBS (HS00609296) expression, which showed higher stability than other common housekeeping genes in the pituitary tissue²²⁹. All of the gene expression assays consist of a pair of unlabelled PCR primers and probes with different dye labels on the 5' end and a nonfluorescent quencher at the 3' end (Figure 3.3).

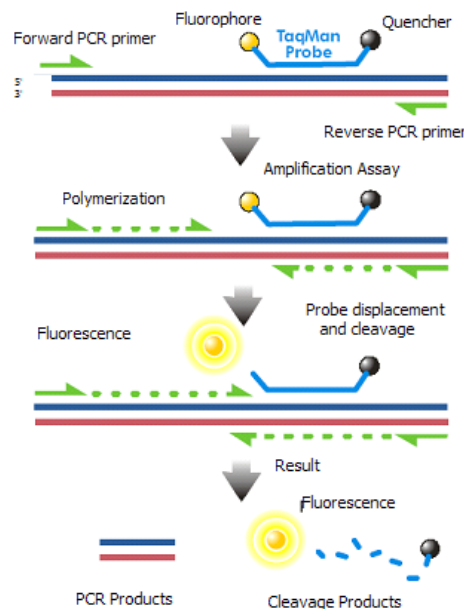


Figure 3.3: Taqman Probe chemistry mechanism: TaqMan probes consist of a fluorophore covalently attached to the 5'-end of the oligonucleotide probe and a quencher at the 3'-end. When the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals. Probes anneal within a region of DNA amplified by two primers. During the PCR reaction, cleavage and consequent degradation of the probe by the exonuclease activity of the Taq polymerase releases the fluorophore from it, allowing fluorescence of the fluorophore due to deactivation of the quenching effect. The fluorescence detected from the thermal cyclers is directly proportional to the template present in the PCR. Taken from <http://www.wikipedia.com>.

qPCR experiments were performed following the MIQE guidelines²³⁰. All samples were assayed in triplicates using a MicroAmp 96-Well reaction plate sealed with an Optical Adhesive Film (Applied Biosystems) using 50 ng of rat cDNA or 20 ng of human cDNA template in a reaction mixture of 20 μ l. No template controls were included with each run as negative controls. In order to check the efficiency of the PCR reaction a standard curve with different dilution of a given cDNA sample has been generated²³¹. qPCR conditions were according to the following scheme: 95°C for 2 minutes (denaturation), 45 cycles at 95°C for 15 second (annealing/extension), 60°C for 1 minute (dissociation). Data were analysed with the Sequence Detection Software 2.4 (Applied Biosystems) with an automatically-set baseline and a fluorescence threshold adjusted for measuring Cq values. Evaluation of gene expression results has been performed using the $2^{-\Delta\Delta CT}$ method²³¹.

3.9 Protein Extraction and quantification (Study I, II)

Protein extraction was performed from GH3 cell lysates in study I to confirm the stable silencing or overexpression of AHRR at protein level and in study II to evaluate the effects of the inhibition of HSP90 activity on HSP90 client proteins. After the removal of cell culture medium cell lysis buffer RIPA with proteases inhibitors (MgCl₂ 10mM, Pepstatin 1 μ M, PMSF 1 mM, Complete 1X (Roche, Monza, Italy)) was added to the cells. Cells were then vigorously scraped from the well and the lysates were transferred on 1.5 ml tubes. Samples were clarified by centrifugation at 13000 rpm for 5 minutes at 4°C and supernatants were stored at -80°C. Bradford assay (Biorad, Hercules, CA, USA) was used for protein quantification. A set of bovine albumin serum (BSA) dilutions was prepared for the standard curve in order to normalise the quantity of proteins loaded for western blot. Protein lysates were diluted 1:250 in distilled water and 100 μ l were transferred in

p96 well plates. 50 µl of a 1:2 solution of Bradford staining diluted in distilled water were added to each well. The plate was then inserted on the reader and absorbance was read at 595 nm wavelength.

3.10 Western Blot (Study I, II)

The Western Blot is a biochemical method that allows a qualitative and semi-quantitative evaluation of proteins and the effect of different conditions on protein levels. It was performed in study I for testing the modulation of the expression of AHRR in stable cell lines and in study II for determining the effects of the treatment with HSP90 inhibitors on client proteins.

For each sample, 20 µg were resuspended in NuPAGE SDS sample buffer (Invitrogen) and NuPAGE sample reducing agent (Invitrogen), boiled for 10 min at 70°C and resolved by SDS-PAGE on 4–12% NuPAGE gels (Invitrogen) and MES buffer (Invitrogen). Separated proteins were transferred onto nitrocellulose membrane by Trans-Blot Turbo transfer system (BioRad). Membrane blocking was performed for 2 hours with 5% non-fat dry milk (BioRad). The membrane was incubated overnight at 4°C with the primary antibody (a list of the primary antibodies used in this thesis is provided in table 3.1). After incubation, the membrane was washed 3 times for 5' with 0.1% Tween 20 (Sigma-Aldrich) in phosphate-buffered saline (Sigma-Aldrich) (TPBS) and incubated for 1h at room temperature with the relative secondary antibody conjugated with Horseradish Peroxidase (HRP) (a list of the secondary antibodies used is provided in table 3.2). Expression was corrected for differences in protein loading by probing blots for 1 h at room temperature with anti-GAPDH antibody (Study I) or rat anti-β actin antibody (Study II) and subsequently with their relative secondary antibodies as previously described. After the incubation with the secondary antibody, the membrane was washed 3 times for

5'with TPBS and incubated with chemiluminescent substrate ECL (Thermo Scientific) for 1'minute. The detection and density measurements were then performed with Chemidoc MP System (Biorad) and Image Analysis Software (Biorad).

Antibody	Dilution	Host	Type	Manufacturer	Study
Anti-AHRR	1:1000	rabbit	polyclonal	ABCAM, Cambridge, UK	I
Anti-CREB	1:2000	mouse	monoclonal	Cell signalling, Danvers, MA, USA	II
Anti-P CREB	1:1000	rabbit	polyclonal	Cell signaling	II
Anti-HSF1	1:1000	rabbit	polyclonal	Novus Biologicals, Littleton, CO, USA	II
Anti-PIT-1	1:1000	rabbit	monoclonal	Santa Cruz Biotech, Santa Cruz, CA, USA	II
Anti-AKT	1:5000	rabbit	polyclonal	Cell signaling	II
Anti-GAPDH	1:10000	mouse	monoclonal	Chemicon, Billerica, MA, USA	I
Anti-β Actin	1:5000	mouse	monoclonal	Millipore, Billerica, MA, USA	II

Table 3.1: List of primary antibodies used in studies I and II:

Antibody	Dilution	Host	Conjugated	Manufacturer	Study
Anti-Rabbit	1:1000- 1:2000	goat	Horseradish Peroxidase	New England Biolabs, Ipswich, MA, USA	I, II
Anti-Mouse	1:1000- 1:5000	horse	Horseradish Peroxidase	New England Biolabs, Ipswich, MA, USA	I, II

Table 3.2: List of secondary antibodies used in studies I and II.

3.11 Cell viability assay (Study I)

Cell viability was measured for evaluating the impact of the modulation of the expression of AHRR. For this purpose the MTT assay was performed, which is a colorimetric assay that detects the conversion of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich) to formazan, as an indication for active metabolic activity. Briefly, 2.5×10^3 cells were seeded in 96-well plates. Every 24h for 4 days after cell seeding viability was measured by adding 5mg/ml of MTT solution to each well. After incubation for 3 h at 37°C, the medium was removed and 100 μ l of DMSO were added to each well and gently mixed to solubilize formazan crystals. Absorbance was measured at 550 nm with a microplate reader (Victor 3 1420 Multilabel Counter, Perkin Elmer) with background subtraction set at 620 nm.

3.12 Caspase 3-7 assay (Study I)

The activity of the effector caspase 3/7 was measured in study I to determine the possible effect of the modulation of AHRR expression in stable cell lines on the induction of apoptotic mechanisms. In this case Caspase-Glo 3/7 assay (Promega, Madison, WI, USA) was used, a luminiscent assay that measures caspase 3-7 activities in cultured cells. Briefly, the assay provides a luminogenic caspase 3/7 substrate, which contains the tetrapeptide sequence DEVD. The addition of this reagent to the cells results in caspase 3/7 cleavage of the substrate and generation of a stable luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase activity present.

Cells were seeded on 96-well plates (2.5×10^4 /well) 24 hours before the experiment. The following day apoptosis was induced with 8h treatment with the apoptosis

inducer Camptothecin (Sigma-Aldrich) at 1 μ M concentration, using DMSO as vehicle. After the treatment early apoptosis was determined by measuring photon emission with a microplate reader (Victor 3 1420 Multilabel Counter, Perkin Elmer, Monza, Italy) following the addition of the appropriate substrate according to manufacturer's instructions.

3.13 Flow cytometric analysis of cell cycle (Study I)

Among its different applications, flow cytometry permits to define different cell types in a heterogeneous cell population and analysing cell size and volume. It is mainly used for measuring fluorescence intensity produced by fluorescent-labeled antibodies detecting proteins, or ligands that bind to specific cell-associated molecules. One of the important applications of this technique is the evaluation of cell cycle population by using a propidium-iodide staining. The latter, is a nucleic acid intercalating agent and a fluorescent molecule that intercalates between the bases of DNA in proportion to the amount of DNA present in the cell; in this way, different intensities of fluorescent signal will permit to distinguish between cells in G1, S or G2 phase.

Cytofluorimetric analysis was performed in study I for determining eventual differences in the distribution of cell cycle population after the stable modulation of the expression of AHRR. Briefly, 1,2X10⁵ cells/dish were seeded in 56 mm diameter Petri dishes. After 24 and 48h of incubation, cells were rinsed in PBS and trypsinized. Trypsin action was then inactivated and the final solution was centrifuged at 1200 rpm for 4 minutes. Subsequently, cell pellet was resuspended in 10 ml cold PBS. After a second centrifugation at the same time and speed as before, the pellet was furtherly dissolved in 1 ml of cold PBS and fixed with 3 ml of ethanol 100% kept at -20°C till used. Cells were then maintained for at least 24h at

-20°C, until propidium iodide staining. Fixed cells were washed in 1ml cold PBS and the pellet was resuspended in 500 µl PBS with 10 µg/ml propidium iodide and 1 µg/ml RNase A for 1h at 37°C, followed by analysis in Beckman–Coulter EPICS XL (Beckman Coulter, Pasadena, CA).

3.13 Senescence associated beta galactosidase assay (Study I)

Senescence-associated beta galactosidase (SA-β Gal) assay is a cytochemical assay that detects the senescence-associated activity of beta galactosidase through the production of a blue precipitate that results from the cleavage of the chromogenic substrate X-Gal⁹⁸. Potential applications of this assay include determining the abilities of different conditions or compounds to induce a senescence response or antiaging effects of compounds in culture or in tissue samples, or determining the appearance of senescent cells after different types of stress.

Senescence-associated beta galactosidase assay was performed for evaluating the possible influence in inducing cell senescence of the stable silencing of the expression of AHRR. 3×10^5 GH3 cells were seeded in 6-well plates. After 48h of incubation, assuring the absence of a sub-confluent state of growth (confluent cells might show aspecific SA-β Gal activity²³²) cells were washed for 30'' with 1ml PBS/well. Cells were then incubated for 5' in fixation solution (2% formaldehyde + 0,2% glutaraldehyde in PBS, 1ml/well). Subsequently, fixation solution was removed for adding staining solution, which consists as reported as follows:

-40mM citric acid/phosphate buffer

-5mM potassium hexacyanoferrate III

-5mM potassium hexacyanoferrate II

-150mM sodium chloride

-2mM magnesium chloride

-1 mg/ml X-Gal

-distilled water in order to reach the suitable volume.

Maximal staining was reached after 12-16 h incubation at 37°C in a non CO₂ atmosphere. Subsequently, cells were washed two times for 30'' with 2ml PBS/well. The last solution used for washing was kept and cells were stored in the dark at 4°C. Staining was then analysed with an optical microscope and quantification of SA-β Gal activity was performed with LAS v1.4 software (Leica Microsystems, Heerbrugg, Switzerland).

3.14 Immunohistochemistry (Study II)

Immunohistochemistry (IHC) is a powerful method for detecting specific antigens in formalin-fixed, paraffin-embedded tissues on antigen-antibody interaction. This method was used in study II for detecting HSP90 overexpression in patients with GH-secreting tumors. Sections of 8 μm size of frozen tissues were cut in a cryostat (Leica CM3050 S, Leica, Wetzlar, Germany) and put on poly-L-lysine coated glass slides. The tissue was fixed in freshly prepared cold PBS with 4% paraformaldehyde (PFA), dehydrated and stored at 4°C until use. At the moment of the experiment, sections were incubated for 5' in Tris-Buffered Saline solution (TBS) at pH 7.6, followed by 30' blocking in TBS with 10% goat serum. Endogenous peroxidase activity was blocked by 15' incubation with TBS and 1% H₂O₂. The sections were incubated with HSP90 antibody (EPR3953 1:200, Epitomics, Burlingame, CA, USA)

diluted in 3% BSA-TBS overnight at 4°C. After three washes of 5' in TBS, the biotinylated secondary antibody (BA 1000 1:500-Vector Laboratories, Burlingame, CA, USA) was diluted in blocking solution and incubated for 30' at room temperature. After three washes in TBS, the slides were incubated for 30' in with the ABC complex (Vectastain ABC Kit, Vector Laboratories), which was prepared 30' prior to use in Tris Buffer to allow complex formation. After 3 washes in TBS, the slides were immersed in freshly prepared DAB reagent (1mg/ml in distilled water) supplemented with 0.01% H₂O₂ until the signal was clearly visible on eye. Each section was incubated in parallel without primary antibody in order to check the background signal. Only the sections which did not have signal in the negative control were considered for the analysis. After three more washes in TBS slides were then counterstained in toluidine blue (1mg/ml in distilled water), which stains the nuclei in order to favour the perceptibility of tissue organization. Any excess of staining was removed by immersing the slides in a 70% ethanol supplemented with acetic acid solution, followed by dehydration and fixation in xylol. Slides were then coverslipped with Entellan (VWR, Radnor, PE, USA) and evaluated at the optical microscope.

3.15 Luciferase gene reporter assay (Study I, II)

Gene regulation can be studied with reporter assays, which permit to evaluate the impact of regulatory elements, on gene transcription. The regulatory elements include potential promoters, known promoters, partial promoters, enhancer elements located on the 5' untranslated region (UTR), upstream open reading frames (uORFs), or regulatory elements located in the 3' UTR region of their gene. The first step of the reporter assay consists in cloning the regulatory element into a vector containing the reporter gene, which encodes a protein for which the activity

is easily measurable (e.g. luciferase). The second step consists in the transfection of cells with the generated plasmid, following the detection of the expression of the reporter protein, which will be proportional to the activity of the regulatory region. Co-transfection with a plasmid constitutively expressing another protein (e.g. β -Gal) is needed in order to normalize the variability of the results due to the efficiency of the transfection or differences in cell growth.

In this case, reporter assays were performed using as the Firefly luciferase reporter gene, which protein emits light when its substrate luciferine is added. A simple representation of this principle is reported in Figure 3.4.

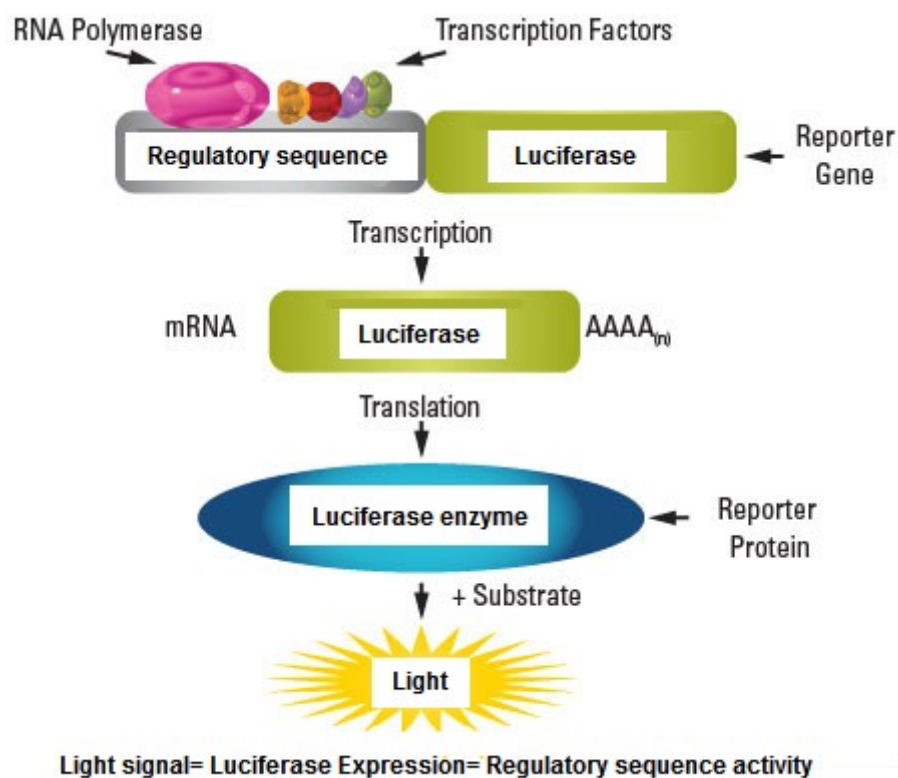


Figure 3.4: Schematic of the luciferase reporter assay. When inserted in the cells, transcription of the reporter gene (luciferase) is starting under the control of the regulatory sequence (in this case upstream of the reporter gene). mRNA is subsequently translated into the reporter protein (Luciferase enzyme); by adding the substrate (luciferine) the reporter protein catalyses a reaction in which the light is emitted: this is the final signal that is be detected, which is proportional to the activity of the regulatory sequence. Adapted from <https://www.thermofisher.com>.

All the plasmids reported were kindly received as a gift, and a list of the sources is provided in appendix 1.

In study I, a plasmid containing XRE upstream of the Firefly luciferase²³³ gene was used for evaluating the activity of XRE-responsive genes in relation to the modulation of the expression of AHRR.

In study II, the effect of the inhibition of the activity of HSP90 was evaluated by reporter assays by using a plasmid containing rat-GH promoter, rat-PRL promoter, CRE promoter or PIT-1 promoter upstream the Firefly luciferase gene.

A plasmid containing β -GAL gene as was used in both the studies for the normalization of the results.

Briefly, 24 h before the experiment, 1×10^5 cells were seeded in 48-well plates. Cells were transfected according to manufacturer's recommendations using 2.2 μ l of Superfect (Qiagen) and 0.4 μ g of total DNA (consisting on the reporter plasmid and β -Gal plasmid for normalization of the results). The day after transfection treatments were performed where needed and at the end of the experiment medium was removed and proteins were harvested in Passive Lysis Buffer (Promega). The protein lysate was then used for evaluating the activity of the reporter by adding the substrate Luciferine (p.j.k, Seoul, Korea) and luminescence indicative for luciferase activity was measured with the Tristar LB941 Microplate Reader (Berthold Technologies, Bad Wildbad, Germany). A buffer containing o-nitrophenyl-b-D-galactopyranoside (ONPG) (Sigma-Aldrich) was added to the lysate for evaluating the activity of β -Gal and measurements were performed with the same microplate reader by reading absorbance at 595 nm wavelength.

3.16 Radioimmunoassay for GH and PRL (Study I, II)3.

Radioimmunoassay (RIA) is an immunological assay for the quantification of the amount of antigen in a sample. The basic principle of radioimmunoassay is competitive binding, where a radioactive antigen named competes with a fixed number of antibodies or receptor binding sites. This assay has been used in study I for evaluating the effect on GH and PRL secretion of the silencing of AHRR and in study II for evaluating GH secretion in GH3 cells in response to the treatment with different HSP90 inhibitors.

Cells were seeded into 96-well plates (2.0×10^3 cells/well in study I; 1.5×10^4 cells/well in study II) in DMEM 10%. After overnight recovery, the medium was replaced with DMEM 2% (Study I) and where needed cells were treated with HSP90 inhibitors (Study II) for the established time. At the end of each incubation time, the supernatants were transferred into a 96-well plate and frozen for further analysis. At the end of the treatments MTT assay was performed in order to normalize GH and PRL secretion. Supernatants were used for RIA analysis as elsewhere reported²³⁴ in the Clinical Neuroendocrinology Unit of Max Planck Institute for Psychiatry, Munich, Germany.

3.17 Statistical analysis

Statistical analysis was performed using the Student's t-test, Kruskal Wallis or Mann-Whitney tests. All tests were completed using the Statistical Product and Service Solutions (SPSS) software program, version 16.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered as statistically significant.

4. Results

4.1 Study I

4.1.1 *In vitro* modulation of AHRR expression

Stable clones were generated from GH3 in order to evaluate the effects of AHRR expression modulation on malignant tumor phenotypes. Two cell lines (shAHRR1 and shAHRR2) were generated with the permanent integration in the genome of different shRNA sequences predicted to have at least 70% efficacy for silencing AHRR at transcriptional level²³⁵. A cell line with the integration in the genome of a scrambled sequence was established as a control (shControl). Another cell line (overAHRR) was created for overexpressing AHRR with the stable integration of a plasmid containing the gene under regulation of the constitutive promoter CMV. Cells with stable integration of the empty vector were used as control. AHRR expression in stable clones was tested at mRNA level by qPCR (figure 4.1 A, B). Stable AHRR-knockdown transfection showed an 81% and 70% downregulation in SHAHRR1 and SHAHRR2 respectively, in comparison to non-silenced shControl ($P < 0.05$). Conversely, after stable AHRR-overexpression a 5.45-fold increase in mRNA expression was observed in comparison to control ($P < 0.05$). Analysis of protein expression by western blot confirmed the qPCR results. AHRR expression was reduced in shAHRR1 and shAHRR2 while overAHRR showed an increased AHRR expression in comparison to Control (Figure 4.1 C).

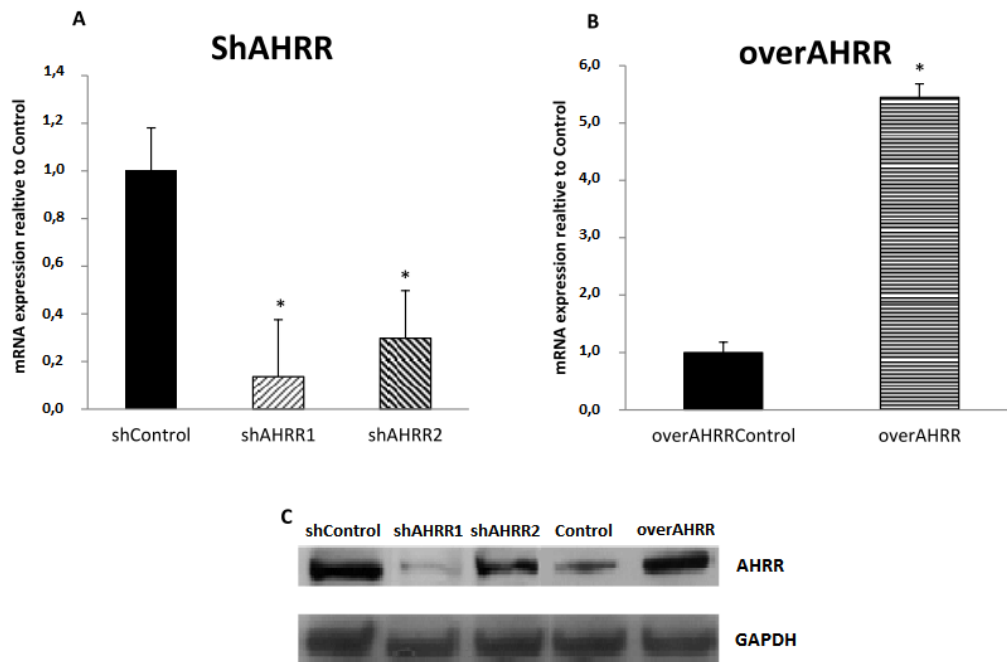


Figure 4.1: AHRH expression of stable cell lines in the GH3 cell line. (A) Downregulation of AHRH mRNA expression was observed in shAHRH1 and shAHRH2 in qPCR in comparison to their control. (B) An increased expression of AHRH mRNA was observed in overAHRH in comparison to its control. Data are presented as a ratio of mRNA levels relative to the respective control and are represented as mean±standard deviation (SD) (n=3) with significant differences at *P<0.05. (C) Western blot analysis confirmed at protein level the qPCR results. GAPDH was detected as loading control.

4.1.2 AHRH expression modulates AHR and XRE-responsive genes activity

The main function of AHRH is to regulate the activity of AHR through a feedback negative mechanism that involves the binding on XRE sequences. (see paragraph 1.4.2.5). In order to evaluate if AHRH expression modulation affects AHR activity, a luciferase reporter assay was conducted by transfecting cells with a plasmid in which the XRE was controlling the expression of Firefly luciferase gene. Luciferase experiments showed an increase in the activity of XRE in both silenced cell lines in comparison to shControl (1.39 and 8.11 respectively, P<0.05) (Figure 4.2A, B);

conversely, a decrease in XRE activity was noticed when AHRR was overexpressed (0.31, $P < 0.05$) (Figure 4. 2C). These data confirm that where AHRR is downregulated, it does not impede AHR binding to XRE, and transcription of XRE-controlled genes finally increases.

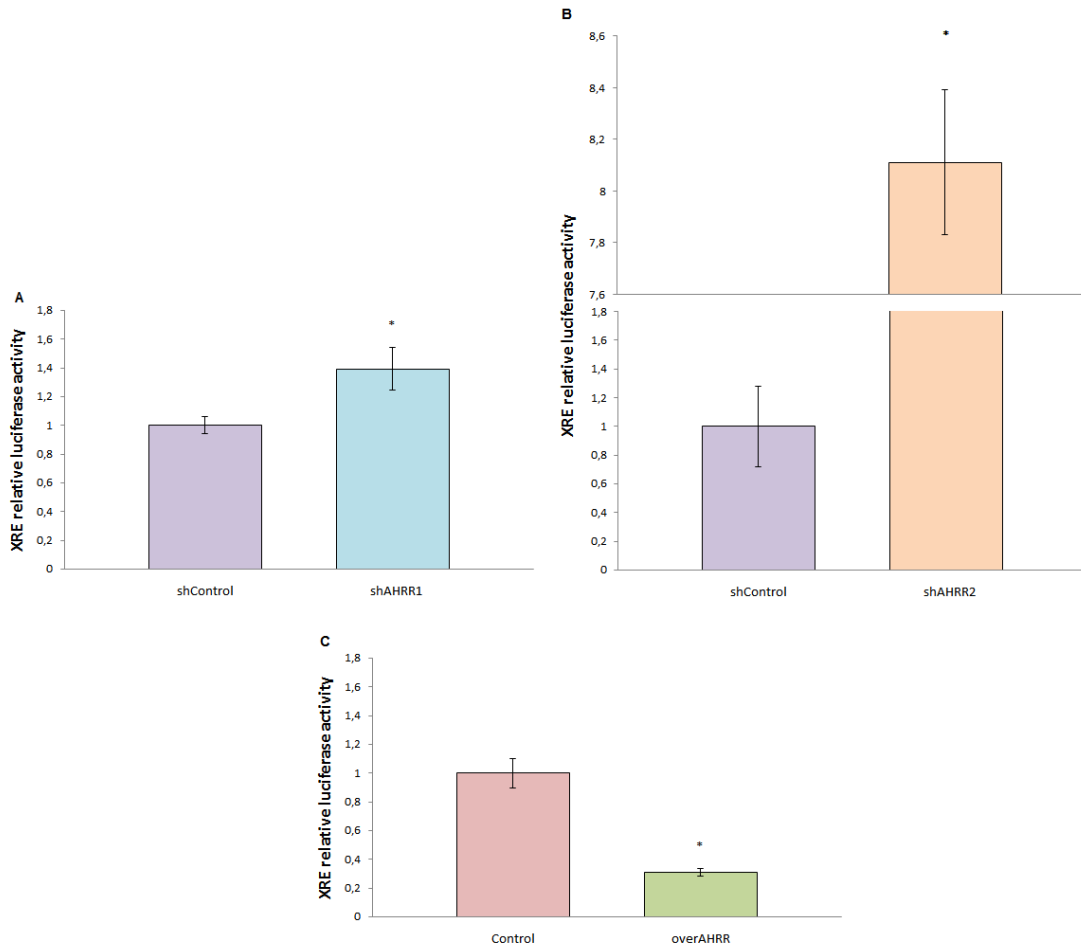


Figure 4.2: Effect of the modulation of AHRR expression on the activity of XRE promoter. Stable clones generated in GH3 cell line were transfected with plasmid containing the XRE sequence upstream of the coding sequence of Firefly luciferase. (A, B) Silencing of AHRR increases luciferase activity in comparison to the unsilenced control. (C) AHRR overexpression decreases the activity of XRE sequence compared to the empty vector control. Data are presented as mean \pm SD in a representative experiment (n=2). *= $P < 0.05$.

4.1.3 AHRR expression influences tumor growth potential *in vitro*

MTT assay was performed for evaluating in stable clones the effect of the modulation of AHRR expression on cell viability. AHRR downregulation resulted in reduced cell growth potential. After a 96 h-time course assay, shAHRR1 and shAHRR2 showed a decrease in viability of 33% and 31% ($P < 0.05$) respectively, compared to the nonsilenced shControl (Figure 4.3 A, B). Conversely, the overexpression of AHRR in the stable line overAHRR led to an increase in viability, which levels were above 61% of the respective control (Figure 4.3 C).

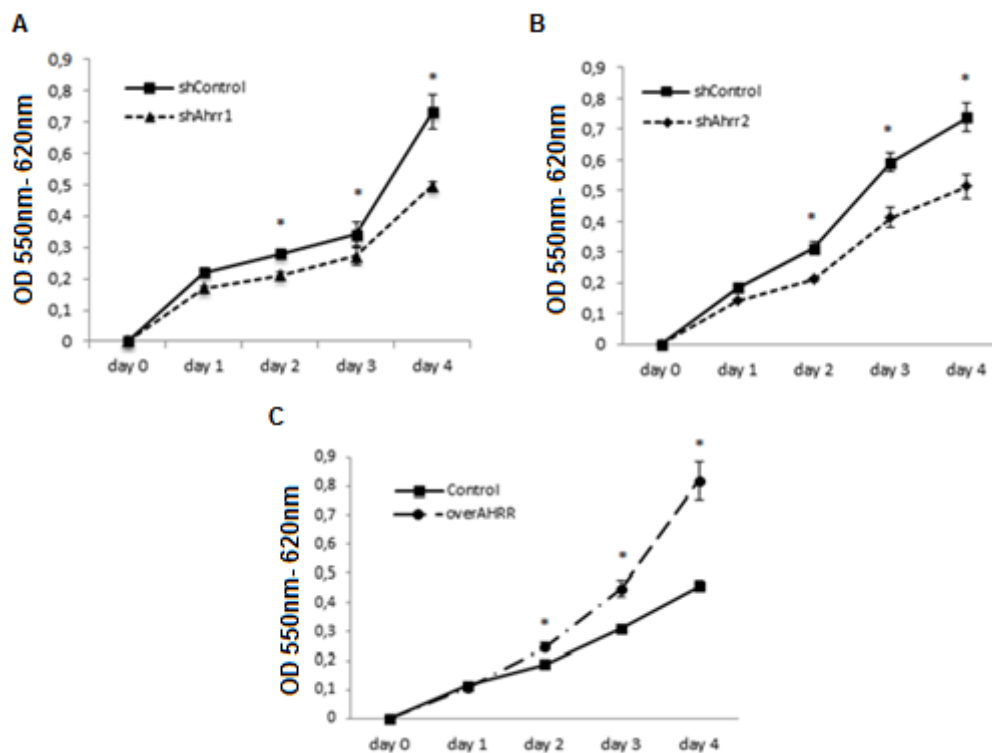
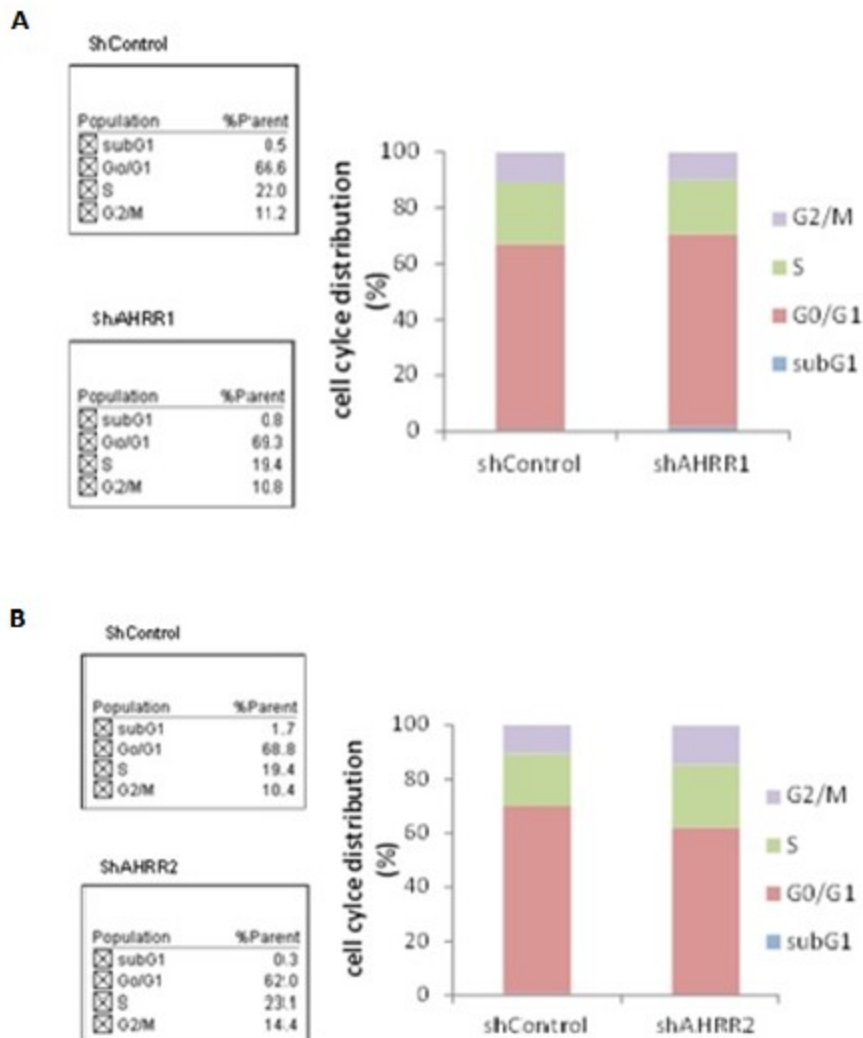


Figure 4.3: 96h-time course MTT viability assay on stable cell lines. shAHRR1 and shAHRR2 (A and B, dashed lines) grew slower than nonsilenced shControl (A and B—continuous lines), respectively 31% and 33%. OverAHRR (C, dashed line) showed a 61% increase in proliferation compared with Control (C, continuous line). Results are representative of three independent experiments. Significant differences are determined by a two-tailed t-Student test ($*P < 0.05$).

4.1.4 Cell cycle distribution is not altered by the modulation of AHRR expression

To determine if the differences in the growth potential of the stable silenced cell lines could be mediated by a change in cell cycle distribution, cytofluorimetric analysis was performed on shAHRR1 and shAHRR2 and overAHRR, after propidium iodide staining. Nor the silencing of AHRR neither its overexpression showed to induce any shift in cell cycle distribution in comparison to the respective controls (Figure 4.4).



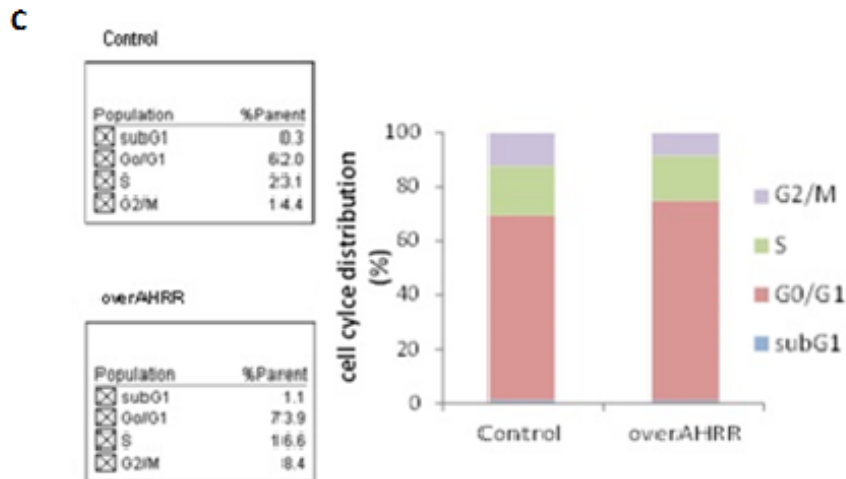


Figure 4.4: Analysis of cell cycle distribution on stable cell lines. Flow cytometric analysis on propidium iodide-stained cells. The percentage of cells in different phases of the cycle are reported in the boxes (A, B, C on the left) and represented by histograms (A, B, C on the right). The results shown are representative of two independent experiments.

4.1.5 AHRR influences resistance to apoptosis

To determine if the modulation of AHRR expression in GH3 cells influences the resistance to apoptosis, caspase 3/7 activity was assayed in stable cell lines under basal conditions and in the presence of the apoptotic inducer camptothecin for 8 hours. In the AHRR silenced cell line shAHRR1 caspase 3/7 activity was higher both in absence and in presence of the apoptotic stimulus in comparison to shControl (1.42-fold and 53.66-fold respectively, $P < 0.05$) (Figure 4.1A). The effect of the treatment in inducing caspase 3/7 activity was higher in shAHRR1 than in shControl (2.48-fold, Figure 4.5D). ShAHRR2 did not display a different caspase 3/7 activity in comparison to its control under basal conditions, whether camptothecin induced a 4.84-fold increase of caspase 3/7 activity in comparison to shControl ($P < 0.05$, Figure 4.5B). As showed by the previous AHRR-silenced cell line, shAHRR2 displayed a higher effect of camptothecin in increasing caspase 3/7 activity than

shControl (1.67-fold, Figure 4.5D). Contrary to the effect of AHRR silencing, stable overexpression of AHRR showed a decreased activity of caspases 3/7 that was similar both in presence and in absence of the apoptotic stimulus (0.6-fold, $P < 0.05$) (Figure 4.5C, D).

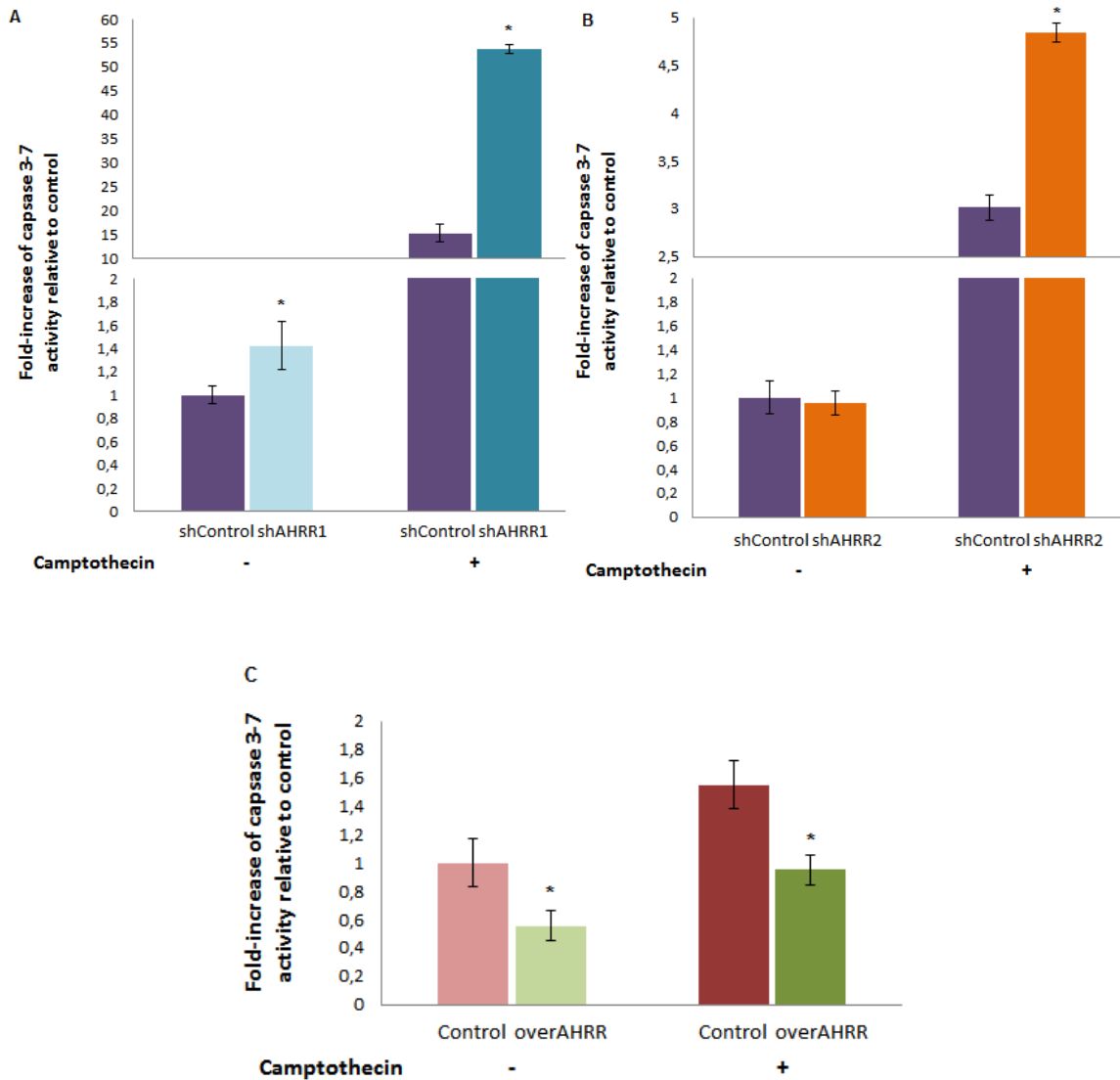


Figure 4.5: Effect of the modulation of AHRR expression on the resistance to apoptosis..

ShAHRR1 (A) and shAHRR2 (2) showed an increase in sensibility to apoptotic stimuli respectively. Conversely, overAHRR (C) was more resistant to apoptosis independently from the presence of the stimulus in comparison to Control. Data are presented as mean \pm SD in a representative experiment (n=2). *= $P < 0.05$.

4.1.6 AHRR downregulation decreases growth hormone and prolactin secretion

GH and PRL secretion were evaluated in stable cell lines in order to clarify if AHRR expression modulation could alter hormonal secretion that is considered a pituitary malignant marker, together with increased proliferation and increased resistance to apoptosis. ShAHRR1 cells were seeded and supernatants were collected after 1h, 6h, 24h and 48h for GH and PRL secretion quantification by RIA. shAHRR1 showed a decreased GH secretion compared to the control, with maximal effect reached at 48h (Figure 4.6A). Moreover, AHRR-silenced cells showed a decrease in PRL secretion in all the time points in comparison to the control (Figure 4.6B).

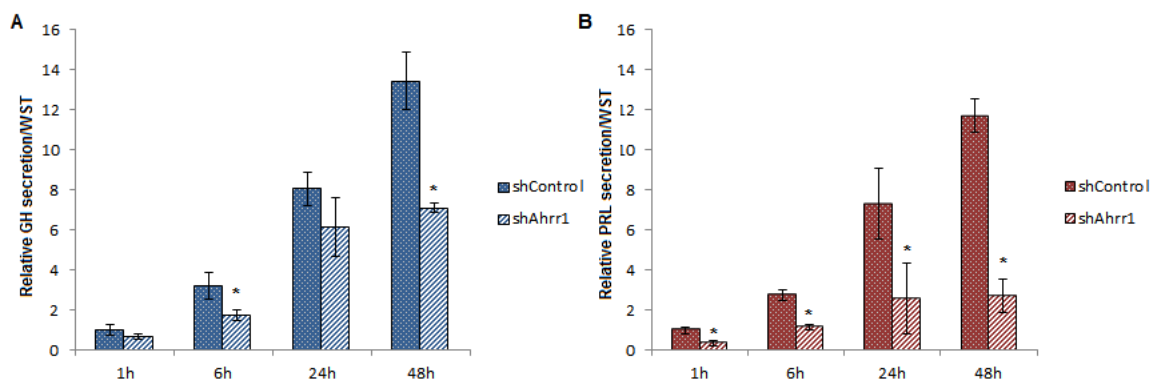


Figure 4.6: Effect of AHRR silencing on GH and PRL secretion. A time-course experiment was performed for quantifying GH and PRL secretion in the stable clone shAHRR1 and its respective control. AHRR silencing decreases GH (A) and PRL (B) production in comparison to shControl with maximal effect after 48h of incubation. Hormonal secretion values reported were normalized for the values obtained by running the MTT assay at the moment of the removal of the supernatant. Data are presented as mean \pm SD (*=P<0.05).

4.1.7 Evaluation of the expression profile of AHRR in human pituitary adenomas

The cohort consisted in 51 patients, 40 were diagnosed with acromegaly (35 with GH oversecretion, 5 with GH and PRL oversecretion), 11 with non-secreting adenomas; 6 normal pituitaries were tested in this study. The expression of AHRR was evaluated in all the patients by qPCR. Values were normalized considering one sample as calibrator and analyzed with the $-2^{\Delta\Delta Cq}$ method (see paragraph 3.8). Mean values among the different types of pituitary tumors are reported in table 4.1.

	Normal pituitary (n=6)	Acromegaly (n=40)		Non- secreting (n=11)
		GH-secreting (n=35)	GH+PRL-secreting (n=5)	
AHRR	0.1±0.006	0.53±0.72 (ACRO)		0.22±0.18
		0.48±0.55 (GH)	0.63±1.15 (GH+PRL)	

Table 4.1: Analysis of AHRR expression in qPCR. Mean values for AHR, ARNT and AHRR expression (values relative to the calibrator) in normal pituitaries and different types of pituitary adenomas analyzed by qPCR.

No ~~statistical~~ differences in expression of *AHRR* between acromegalic patients and normal pituitaries were found ($p=0.122$). By dividing the group of acromegalic patients in GH-secreting tumors and GH+PRL secreting tumors, GH-secreting tumors showed a higher expression of AHRR in comparison to normal pituitaries ($p=0.041$), whether GH+PRL tumors. No difference in AHRR expression was found

between normal pituitaries and NFPA samples. A representative distribution of the results is showed in the box -plots of figure 4.8.

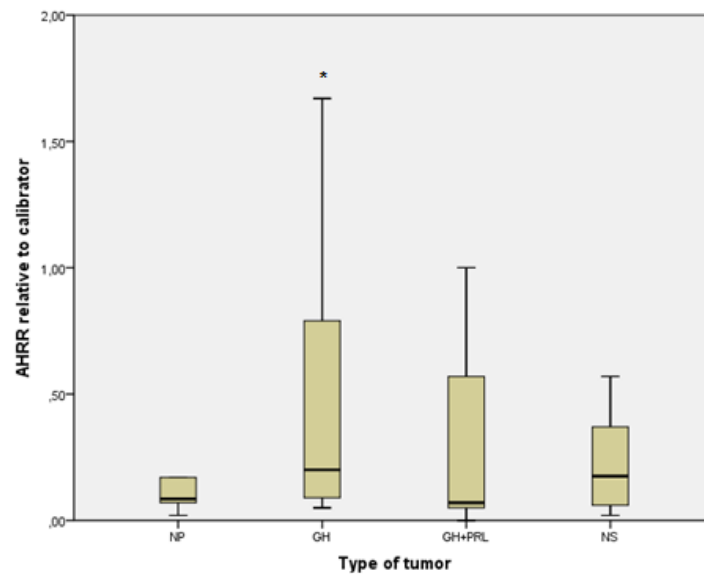


Figure 4.8: Distribution of AHRR expression values among different types of pituitary adenomas. Representative boxplot showing minimum, maximum, median, average and 1st and 3rd quartile values for all normal pituitaries (NP), GH-secreting adenomas (GH), GH+PRL secreting adenomas (GH+PRL) and non-secreting adenomas (NS). GH-secreting tumors showed a higher expression of AHRR in comparison to normal pituitaries (*= P<0.05).

4.1.8 Evaluation of the AHR pathway expression in relation to radiological and pathological parameters of aggressiveness

The expression of AHRR was subsequently analyzed in the different types of pituitary adenomas for patients with available information about radiological parameters of aggressiveness ^{38,39} (Table 4.2). No correlation was found between AHRR expression levels and radiological aggressiveness in acromegalic and NFPA groups.

	Aggressive	Non aggressive	P
Acromegaly (17)	0.552±0.66 (9)	0.842±1.042 (8)	0.7
Non-secreting (11)	0.187±0.148 (8)	0.357±0.226 (3)	0.15

Table 4.2.: AHRR expression in radiologically aggressive and radiologically non aggressive pituitary tumors. Mean values relative to the calibrator and sample size of the patients with pituitary adenomas with or without pituitary tumor aggressiveness.

Statistical analysis did not show any correlation between AHRR expression and pathological parameters of aggressiveness²¹(Table 4.3). No association with AHRR expression levels was found by comparing aggressive and non-aggressive tumors in acromegalic patients (p=0.261) and in patients with non-secreting pituitary adenomas (p=0.374).

	Aggressive	Non aggressive	P
Acromegaly (12)	0.703±0.688 (6)	0.375±0.485 (6)	0.261
Non-secreting (5)	0.263±0.275 (3)	0.060±0.028 (2)	0.374

Table 4.3.: AHRR expression in pathologically aggressive and pathologically non aggressive pituitary tumors. Mean values relative to the calibrator and sample size (within brackets) of the patients with pituitary adenomas with or without pituitary tumor aggressiveness.

4.2 Study II

4.2.1 HSP90 is overexpressed in biopsy specimens of human GH-secreting pituitary adenomas

The excessive expression of HSP90 has been observed in different types of cancer. Considering that it was recently demonstrated its role in the pathogenesis of

Cushing's disease, it was hypothesized that HSP90 overexpression could be implicated in the pathogenesis of other hormone secreting pituitary adenomas. HSP90 immunohistochemical staining was analyzed in biopsy specimens of pituitary adenomas from acromegalic patients that had undergone transphenoidal surgery, using as a control normal pituitary tissue. Intense immunostaining for HSP90 was detected in GH-secreting adenomas from acromegalic patients (n=8/25, scores reported in appendix 2). A representative picture for the cytoplasmic staining is shown (Figure 4.9). The remaining GH-secreting adenomas which were not scored as positive had little or no signal specific for HSP90. Conversely, normal human pituitaries showed a typical morphology with only few single cells weakly expressing HSP90.

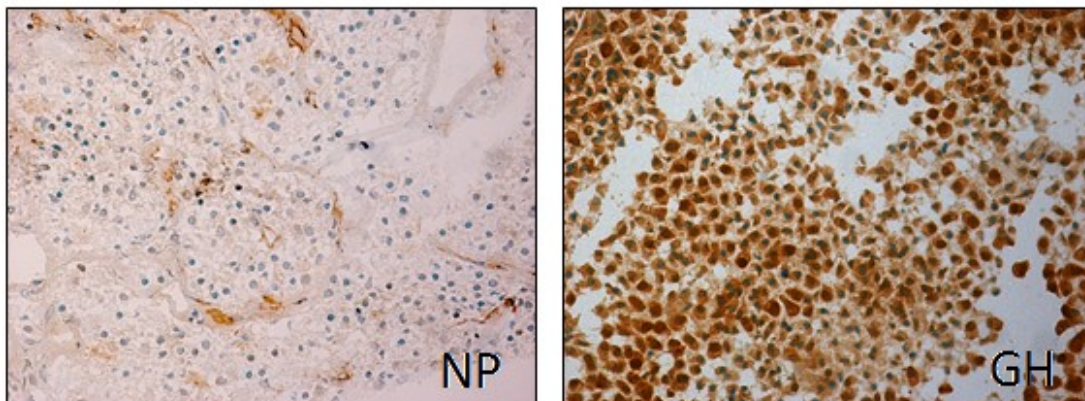


Figure 4.9: Immunohistochemistry of HSP90 in cryosections. A representative example of a human GH-secreting pituitary adenoma (GH) from an acromegalic patient, and post-mortem normal pituitary tissue (NP) is shown. Counterstaining of nuclei was done with toluidine blue. HSP90 α specific signal is brown (DAB). Magnification: 40X.

4.2.2 C-terminal inhibitors of HSP90 novobiocin and KU174 decrease the activity of GH-promoter

Reporter gene assays were performed for evaluating the effect of the HSP90 inhibitors tested on the activity of GH-promoter. GH3 cells were transiently co-transfected with the reporter plasmid and β -gal for normalizing the results obtained. Figure 4.10 displays the effects of 24-hours treatment with the indicated HSP90 inhibitors. The N-terminal inhibitor 17AAG did not decrease the activity of the GH promoter. Conversely, treatment with the C-terminal inhibitor of HSP90 novobiocin decreases in a dose-dependent way the activity of the GH-promoter with maximal luciferase inhibition at the 100 μ M concentration (50%, $P < 0.05$). KU174 resulted to be more efficacious in decreasing the activity of GH-promoter, with maximal luciferase inhibition at 4 μ M (77%, $P < 0.05$).

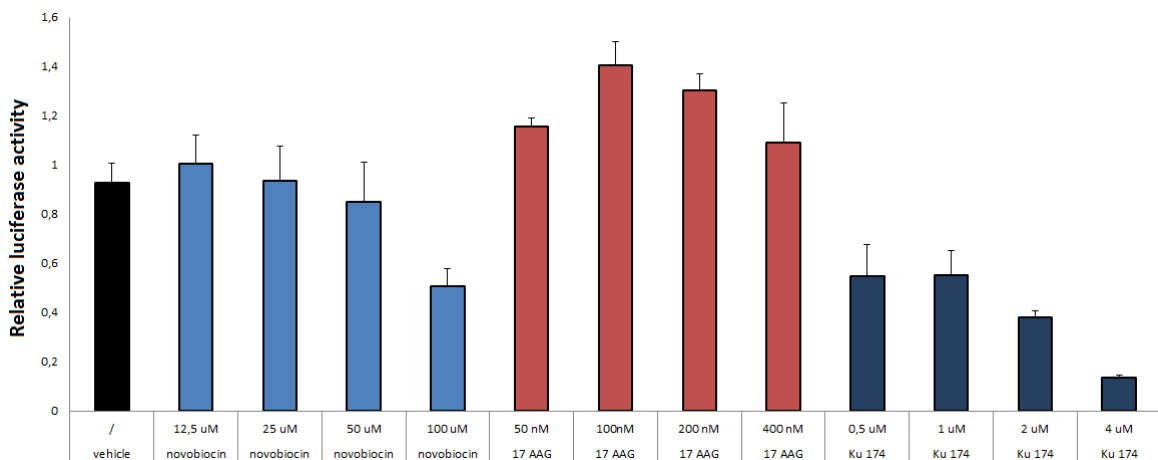


Figure 4.10: Determination of GH-promoter activity in GH3 cells using reporter gene assay.

Effect of 24h treatment with the N-terminal inhibitor 17-AAG and the C-terminal inhibitors novobiocin and KU174 on GH-promoter activity. Values are represented in comparison to the untreated control. Means \pm SD of one representative experiment are shown ($n=3$, $*P < 0.05$).

4.2.3 The C-terminal inhibition of HSP90 KU174 decreases growth hormone secretion in a cellular model of somato-lactotroph pituitary adenoma

In order to investigate the downstream effects of HSP90 inhibition GH3 cells were treated for 24 h with increasing concentrations of 17 AAG, novobiocin and KU174 and supernatants were collected and GH was quantified by RIA. The C-terminal inhibitor KU174 decreased GH secretion levels in a dose-dependent way, being both effective at 2 μ M (28%, P<0.05) and 4 μ M (54%, P<0.05) (Figure 4.11).

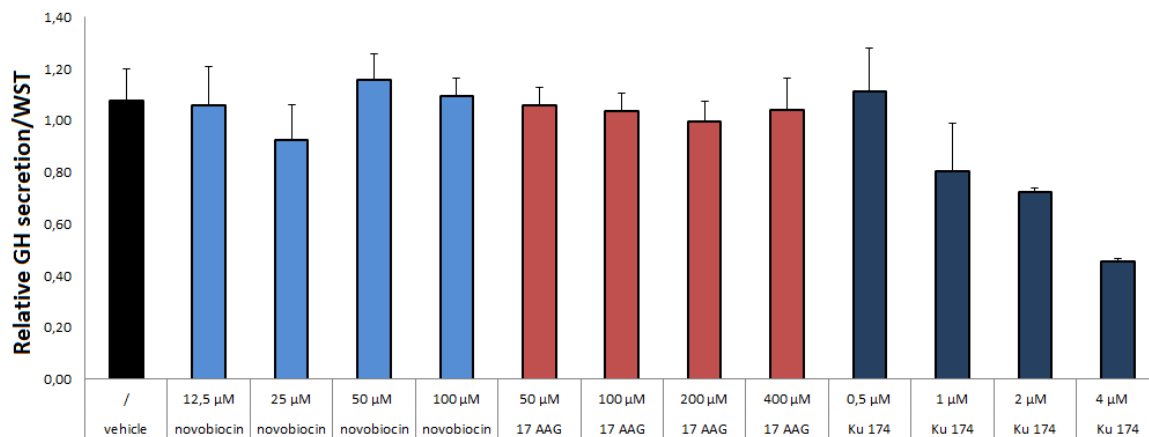


Figure 4.11: Evaluation of the effect of HSP90 N-and C-terminal inhibitors on GH-secretion in GH3 cells. Cells were treated for 24h treatment with the N-terminal inhibitor 17-AAG or one of the C-terminal inhibitors novobiocin and KU174. Values are represented in comparison to the untreated control. Means \pm SD. of one representative experiment are shown (n=3, *P<0.05).

4.2.4 C-terminal inhibition of HSP90 with KU174 decreases the promoter activity of PIT-1 and CRE

The second reporter assay was performed for testing the effect of HSP90 inhibition on the activity of promoters of genes primarily involved in the GH signaling: *CREB* (cAMP response element-binding protein, a cAMP-responsive transcription factor regulating the somatostatin gene) and *PIT-1* (pituitary-specific transcription factor 1, responsible for pituitary development and hormone expression in mammals). GH3

cells were transiently co-transfected with the reporter plasmids and the β -Gal for normalization. Figure 4.12 shows the effect of 6h treatment with the HSP90 C-terminal inhibitor KU174. Consistent with the previously reported results, the drug showed a dose-response effect in decreasing the activity of both promoters in presence of forskolin (FSK) 10 μ M.

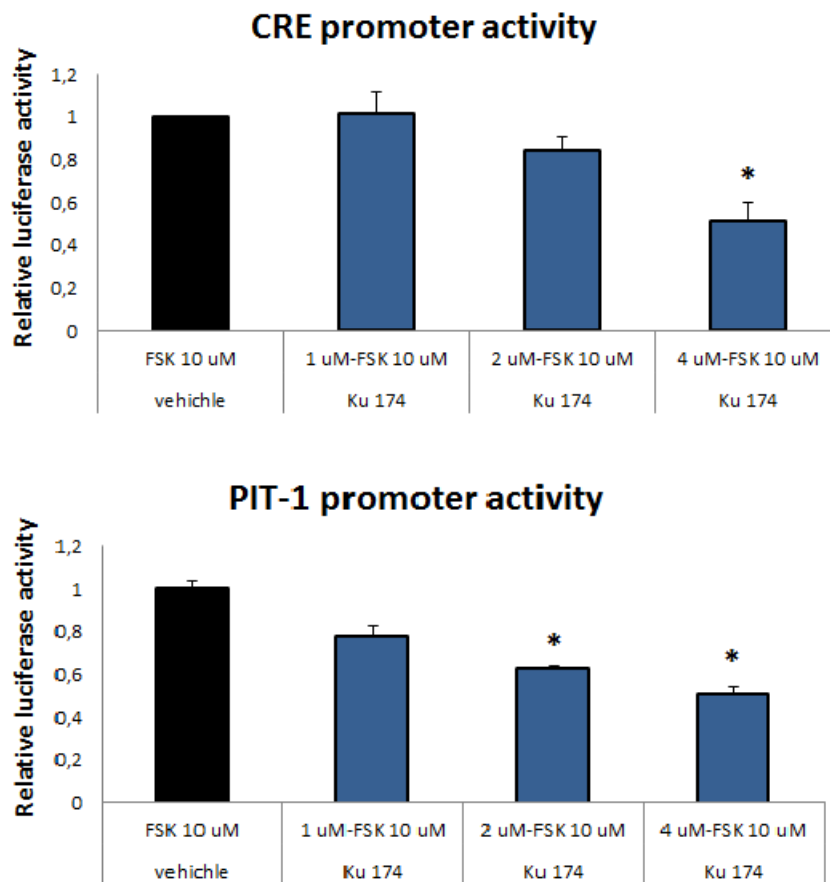
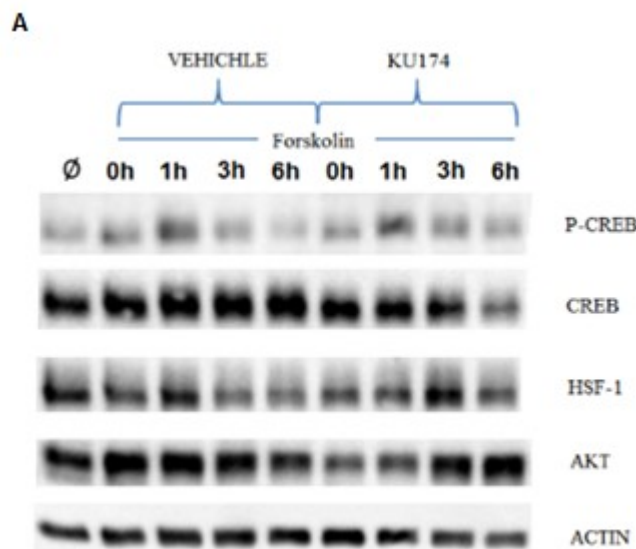


Figure 4.12: Determination of *CRE*-and *PIT-1*-promoter activity in GH3 cells using reporter gene assay. Cells were treated with the C-terminal inhibitor KU174 (in presence of 10 μ M forskolin). The activity of *CRE*-and *PIT-1* promoters was evaluated after 6h of incubation. Values are represented in comparison to the untreated control. Means \pm SD of one representative experiment are shown (n=3, *P<0.05).

4.2.5 Exposure of GH3 cells to KU174 results in downregulation of Akt, CREB and Pit-1 expression

The effect of KU174 on the expression of client proteins of HSP90 and pituitary tumor-related proteins was subsequently examined by western immunoblotting (Figure 5) in absence and in presence of 10 μ M forskolin stimulation. 48h incubation with KU174 at 4 μ M concentration in absence of forskolin stimulation (0h) resulted in a 60% decrease in the expression of Akt (Figure 5B), which is known to be direct interactor of HSP90 and is involved in the activation of CREB protein ¹⁸⁰. A progressive decrease of total CREB expression was registered during the treatment with forskolin with minimal expression after 6h stimulation with forskolin (53% compared to stimulated control, Figure 5C). No increase in HSF-1 expression was reported after during the treatment KU174 as a proof of the absence of the induction of a stress response (Figure 5D).



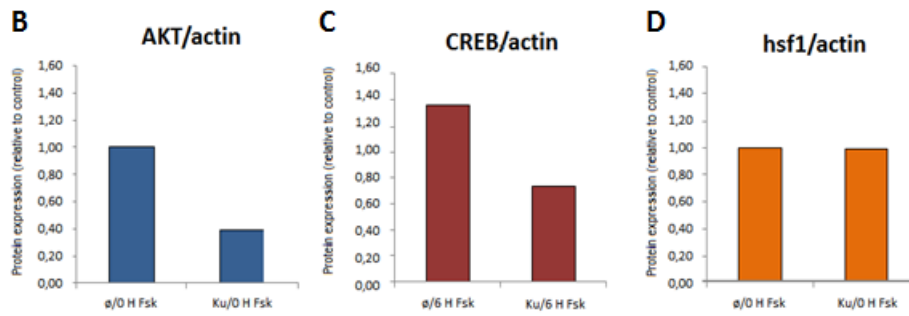


Figure 5: Immunoblot representing the treatment with KU174 and after 1h, 3h and 6h co-treatment with forskolin in GH3 cells. (a) Cells were initially treated for 48h with KU174 (4 μ M) and then stimulated for 6h with forskolin 10 μ M for evaluating the effect of the C-terminal inhibition of HSP90. Actin was used as loading control. (b) Quantification of Akt protein expression after 48h treatment with KU174 4 μ M; (c) Quantification of total CREB expression after treatment with KU174 4 μ M and final 6h stimulation with forskolin 10 μ M; (d) Quantification of HSF1 expression after 48h treatment with KU174 4 μ M.

5. Discussion

5.1 Study I

Several aspects about pituitary tumorigenesis still need to be understood, especially the factors determining the particular benign and slow-growing nature of these tumors; some answers to these questions could be found through the analysis of novel molecular pathways which have been recently considered.

AHR pathway has been recently hypothesized to play a role in pituitary tumorigenesis. In fact, different studies proved the relation between AHR and the modulation of the pituitary function^{135,136,140,142}, and its correlation with pituitary tumor aggressiveness or a more severe disease^{140,142}. However, still little is known about the role of AHRR in the pituitary, apart from the demonstration of its activation after the stimulation of AHR activation, confirming the existence of a functional feedback mechanism of regulation of this pathway *in vivo* and *in vitro*^{158,159}. Considering the few studies reported in literature which proposed AHRR as new candidate TSG^{160–162}, and the presence of AHRR overexpression reported in somatotropinomas¹⁶³, the main focus of this study was to investigate its possible role in pituitary tumorigenesis. Stable cell lines were generated for AHRR silencing or overexpression. The analysis of the XRE reporter activity proved that the modulation of the expression of AHRR potentially influenced the activity of XRE responsive genes, giving an indirect proof of a functional feedback mechanism of regulation of AHR activity and AHR-related genes, in accordance with previous studies^{158,159}. In addition, the differential expression of AHRR showed decreased growth potential, whether AHRR overexpression displayed the opposite effect. The MTT results were confirmed by cell counting (Trypan blue staining, data not

reported). These findings could reflect cell- or adenoma/carcinoma context or tissue-dependent differences in the overall activity of the AHR signaling, including AHRR behavior. To support the fact that AHR-AHRR signaling acts in a tissue dependent manner and that specifically in pituitary AHR activation seems to be correlated with a milder malignant phenotype, treatment with curcumin, and indirect activator of AHR²³⁶, displayed antiproliferative effect in pituitary adenomas both *in vitro* and *in vivo*²³⁷. In a recent report by Formosa et Al. in 2015, AHR overexpression in the GH3 cell line and activation with B α P reduced cell proliferation, which is accordance to the effect on proliferation of the modulation of AHRR expression²³⁸.

AHRR silencing in stable cell lines led to a decreased resistance to the treatment with the apoptosis inducer camptothecin in comparison to the control; conversely, AHRR overexpression showed an increased resistance to the same stimulus. These data provide another proof of the fact that the modulation of the regulation of the AHR-AHRR pathway influences the aggressiveness of the tumor phenotype, as already reported in *in vitro* studies^{140,237,239}.

Due to the interesting results that suggested a role of AHRR in regulating commonly considered hallmarks of tumor activity (cell proliferation and apoptosis), the impact of AHRR silencing in hormone secretion has been further investigated. Considering that GH3 cells are a model for GH- and PRL- secreting pituitary adenomas, the secretory activity of both hormones has been evaluated. As a result of AHRR silencing, cells reduced the secretion of GH and PRL in comparison of control. These results are apparently in contrast with the first reported evidence of Elango et al., 2006 in which the modulation of pituitary function by TCDD in rainbow trout pituitary was investigated, and AHR activation led to an increase of GH and PRL mRNA expression. However, this study also reported a suppression of GH and PRL

expression at very low doses of TCDD in the pM range¹³⁵. Partially in accordance to this study, a more recent study by Moran et Al. in 2012 displayed that co-treatment of GH3 cells with β NF and α NF (which is supposed to have opposite effects of β NF) displayed PRL gene suppression, with little or no effect on GH gene expression¹³⁶. Although the decrease of GH and PRL secretion reported in this study might support the hypothesis that of AHRR silencing may mitigate tumor phenotype of GH- and PRL- secreting adenomas, further evaluations are needed to finely understand the molecular pathways involved in this phenomenon. Even in presence of a possible involvement of XRE-responsive genes modulation demonstrated by reporter experiments in GH3 cells, it is still unclear if the decrease in GH/PRL secretion in AHRR silenced cells is directly mediated by AHRR-AHR signaling or if it involved AHR independent pathways.

In addition, cellular senescence has been evaluated for its possible contribute to the slow growing phenotype of AHRR silenced cell lines. Senescence could partially explain the peculiar behavior of pituitary tumors, which usually are benign present a very slow growth rate⁹⁶. In fact, it is consolidated that cellular senescence represents an important protection against malignancy⁷³. ShAHHR1 showed an intense SA- β Gal staining in comparison to the non-silenced control, in accordance to the lower proliferation rate reported in AHRR-silenced stable cell lines. However, no variations in cell cycle population were found. This result could be partially explained by an uncoupling between cell cycle arrest and the manifestation of cellular senescence due to some molecular mechanisms independent from Cdk activity²⁴⁰.

The aim of the final part of this study was to investigate possible correlation between pituitary adenoma aggressiveness and AHRR expression in acromegalic and

NFPAs patients. Consistent with previous findings, AHRR was overexpressed in patients with GH- secreting pituitary adenomas in comparison to normal pituitaries¹⁶³, whether no differences were found in AHRR expression between prolactinomas and NFPAs in comparison to normal pituitaries. Due to the lack of a clear consensus for the determination of the aggressiveness of pituitary adenomas, tumor aggressiveness was evaluated in the analyzed cohort ~~analyzed~~ referring to the available data regarding pathological aggressiveness (MIB-1 and p53 IHC staining) or radiological aggressiveness (invasion by the tumor of the surrounding anatomical structures). No correlation was found in the selected cohort between AHRR expression and the presence of aggressiveness markers. Moreover, is important to consider that just few data were available regarding tumor aggressiveness in the analyzed cohort, therefore increasing the sample size could improve the consistency of this analysis. Finally, the possible problem of the cellular heterogeneity of surgical specimens could be partially overcome by IHC analysis, which can detect the expression of AHR-AHRR directly on tumor cells. This study is actually ongoing but results are still too preliminary to be discussed herein.

In conclusion, these results provide the demonstration of a putative oncogenic role of AHRR in the GH3 cell line model used by affecting AHR activity. Further evaluations are needed to clarify the molecular mechanisms behind the presence of a senescent phenotype due to AHRR silencing and to confirm the findings observed in the cellular model in human pituitary adenomas.

5.1 Study 2

Increased chaperone expression of the proteins belonging to the HSP family contributes to oncogenesis at several levels¹⁷⁰. In particular, a constitutive and 2-10-fold higher HSP90 expression was reported in various tumor cells in comparison to their normal counterparts, which means that its function must be important for growth and survival of tumor cells^{176,186}. Therefore, greater attention has been put to HSP90 as a possible therapeutic target, and several inhibitors have been designed with promising results in different types of tumors. Recently, the role of HSP90 in ACTH-secreting tumors has been investigated by Riebold et al., 2015. The authors found that HSP90 is overexpressed in corticotroph adenomas in comparison to the normal pituitary, and treatment with C-terminal inhibitors of HSP90, especially silibinin, showed an impressive therapeutic efficacy with a partial recovery of the glucocorticoid sensitivity¹⁷⁷. Based on these findings, aim of this study was to clarify if HSP90 could be implicated also in the pathogenesis of GH-secreting pituitary tumors. IHC analysis of GH-secreting tumors displayed overexpression of HSP90 in 8/25 patients. This result gives some support to the hypothesis that HSP90 expression could be one of the possible mechanisms that drive the pathogenesis of GH-secreting pituitary adenomas. Subsequently, the therapeutic effect of N- and C-terminal inhibitors of HSP90 was tested by using the GH3 cell line as a model. The effect of the different inhibitors on the activity of the GH promoter was tested. The C-terminal inhibitors novobiocin and KU174 decreased GH-promoter activity in a dose-dependent manner. The effect of KU174 was more pronounced, due to the fact that this compound is an improved analogue of the novobiocin²¹⁸. Conversely, the N-terminal inhibitor 17-AAG increased the transcriptional activity of GH, suggesting a different effect mechanism of action on

client proteins possibly due to the inhibition of the N-terminal domain. GH secretion quantification of GH3 cells after the treatment with HSP90 inhibitors confirmed the effects observed at transcriptional level: only KU174 is able to decrease GH secretion in a dose-dependent way, while novobiocin and 17-AAG did not lower GH. While the lack of effect of 17-AAG could be interpreted as the lack of effect in GH-secreting pituitary tumors, the absence of a suppressive effect of novobiocin could be due to its reduced potency for inhibiting HSP90 in comparison to KU174²¹⁸.

Due to these promising results regarding the efficacy of KU174 in terms of GH production and secretion, the molecular mechanisms that could be involved in HSP90 inhibition have been assayed. The effect of HSP90 inhibition has been tested in relation to the activity of CRE- and PIT-1 promoters, which play a pivotal role in controlling the expression of human and rat GH genes¹¹. CRE is a cAMP response elements present on the human GH promoter. CRE is normally target of the proteins related to the cAMP-response element binding protein (CREB)/activating transcription factor-I (ATF-1). After the stimulation with forskolin, which mimicked the the cAMP increase induced by the stimulation of GHRH, a dose-response decrease in the activity of CRE promoter was reported. This result suggests a role of the inhibition of HSP90 by KU174 in controlling the response to high cAMP levels, possibly due to the lack of stabilization of client proteins involved in the cAMP pathway that are interacting with a functional HSP90.

PIT-1 is a pituitary-specific transcription factor regulating the expression of both rat and human GH genes through the binding to its specific sites in the GH promoter region^{11,12}. After the stimulation of the cAMP pathway, a dose-response suppressive effect of the activity of the promoter by KU174 was observed, suggesting that the overall efficacy of the compound could be due to a pituitary-specific effect. Western

blot analysis was then performed in order to verify if these findings could be correlated to protein degradation of pituitary-specific markers and HSP90 interactors. Although no clear effect was observed regarding the amount of phosphorylated CREB, there was a clear decrease in total CREB expression, which could suggest the degradation of the total protein due to the inhibition of the activity of HSP90. Moreover, A decrease expression of Akt protein was also observed, which is a known interactor of HSP90. These two findings could be correlated by the fact that CREB could be a downstream target of the HSP90/Akt signaling, so that the inhibition of the activity of HSP90 could have an impact on CREB expression or stability^{241,242}. Another important finding to consider is that the C-terminal inhibitor KU174 exerted its effect without eliciting a heat shock response because no increase in HSF-1 was encountered. This particular aspect was already reported in a previous study in prostate cancer and could partially explain the reason why C-terminal inhibitors of HSP90 showed a better efficacy in comparison to the N-terminal counterparts^{216,218,243}.

In conclusion, thanks to the promising effects demonstrated by KU174, this study provides the rationale for permitting further investigations on HSP90 as a target for the therapeutic management of GH-secreting tumors. Moreover, it confirms the biological differences already reported in several studies between N- and C-terminal inhibitors of the HSP90, possibly due to the degradation of a different battery of interactors or co-chaperones including the differential effects on HSF-1, which activation with N-terminal inhibitors drives the induction of a heat shock response.

Appendix 1

List of plasmids used in this thesis.

Plasmid	Source/ Kind gift of
shAHRR1	SABiosciences, Frederick, MD, USA
shAHRR2	SABiosciences, Frederick, MD, USA
shControl	SABiosciences, Frederick, MD, USA
overAHRR	ORGene, Rockville, MD, USA
Control	ORGene, Rockville, MD, USA
XRE-luc	Xavier Coumoul, Univ. Paris Descartes, France
rGH-luc	Gutierrez-Hartmann, University of Colorado, USA
CRE-luc	Clontech Laboratories Inc., Palo Alto, CA, USA
PIT-1-luc	C. Alvarez, Univ. of Santiago de Compostela, Spain
β-Gal	D. Spengler, MPI of Psychiatry, Germany

Appendix 2

IHC staining score of the cohort of GH_secreting pituitary adenomas analysed. -= no expression; +/- = low expression; += medium/high expression; +++ = high expression

Sample	Score
1	+/-
2	+/-
3	++
4	+/-
5	+/-
6	+/-
7	+/-
8	+/-
9	-
10	+
11	+
12	+
13	++
14	+
15	+
16	+/-
17	+/-
18	-
19	-
20	+
21	+/-
22	+/-
23	+/-
24	-+/-
25	-+/-

Acknowledgements

This study has been carried out during the years 2013-2016 at the Department of Medicine, Hospital/University of Padua, Italy, and the Max Planck Institute of Psychiatry, Munich, Germany. I'm sincerely grateful to all those who participated in this research work:

-My supervisor Prof. Carla Scaroni and my co-supervisors Gianluca Occhi and Daniela Regazzo for their guidance, support and encouragement during these three years of PhD.

-The head of the endocrinology Division of the Hospital/University of Padua Prof. Marco Boscaro, for letting me the opportunity to work in the intellectually challenging environment of the Adrenal and Pituitary Physiopathology research group.

-Prof. Günter Stalla, for accepting me in its research group as a visiting student during the last year of my PhD and Dr. Marcelo Paez-Pereda, Dr. Marily Theodoropolou and Dr. Jose Luis Monteserin Garcia, for their friendship technical assistance.

- All friends and colleagues from the Medical Semiotics in Padua and from the Neuroendocrinology Unit in Munich for their friendship and support.

-My family, my girlfriend Lilia, and my friends Alessandro, Andrea, Alessio, Claudia, Valeria, Lauro and Mario, for their continuous support and motivational help for reaching this desired goal in my career.

Munich, April 2016

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