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Estrogen/Neuroglobin pathway as pharmacological target for hormone-related cancer

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SUMMARY

In 2000, the third heme-globin, called Neuroglobin (NGB), has been discovered in nervous system. NGB comes up as a hexa-coordinated globin, but its coordination is reversible and controlled by the oxidative state of the cell as well as by phosphorylation. For this reason, NGB has been postulated to act as a redox sensor in the nervous system. Experimental works assess that NGB is a neuroprotective molecule which overexpression could protect nervous system against several insults. This data prompted several laboratories to find compounds able to increase NGB levels. Recent results showed that 17β-estradiol (E2) strongly induces NGB up-regulation by 300% in human neuroblastoma cell line.

17β-estradiol (E2) is the most concentrated and efficient female sex steroid hormone. In premenopausal women, it is produced in the ovary and its levels are significantly high during the reproductive age. E2 controls many aspects of human physiology, including development, reproduction, homeostasis and brain functions through the activity of its receptors, ERα and ERβ. Given this widespread role of E2 in human physiology, it is not surprising that it is also implicated in the development or progression of numerous diseases, which include various types of cancers (breast, ovarian, prostate and endometrial). The main factors that influence E2 effects are the state and the balance of its receptors, which levels are modified in cancer cells. ERα appears to promote the proliferation of breast, gynaecologic cancers and endocrine gland cancer cells. In contrast, ERβ suppresses the proliferation of tumor cells and drives them to apoptosis. Conversely, to what occur in extra-nervous cancer tissues, in the brain ERα- and ERβ-mediated functions converge to a common outcome, neuroprotection.

Previous work performed in our laboratory indicates that E2 *via* ERβ genomic and rapid signals exerts an anti-apoptotic and protective function in neurons by up-regulating and re-allocating NGB into mitochondria. In addition, in hepatocarcinoma and in breast cancer cells, E2 *via* ERα induced the up-regulation and re-localization of NGB into mitochondrial compartment in which the globin counteracts the apoptotic cascade induced by oxidative stress without any further effect on E2-induced cell proliferation, suggesting a specific role of NGB in the apoptotic pathway.

In order to better define the functional role played by NGB in E2-induced cellular effects, this thesis was aimed: (i) to dissect the signalling pathways, important for cancer cell

survival, which up-stream E2-induced NGB over-expression, (ii) to evidence the function played by NGB in E2-related cancer, and (iii) to screen molecules able to interfere with E2-activated pathway *via* ERα, in order to interfere with NGB over-expression in breast cancer cells.

In the first part of this thesis, we demonstrated that NGB levels are high in human breast tumor tissues, confirming a key role of the globin in breast cancer physiology. Moreover, we showed a strong correlation between NGB and ERα. In order to obtain a more precise correlation between NGB level and ERα activation, we considered five breast cancer cell lines, characterized by a different ERα expression. NGB is expressed in all cell lines but E2 dependent NGB up-regulation is maintained only in ERα(+) breast cancer cell lines (i.e., MCF-7, T47D, ZR-75-1); while no E2 effects are reported in ERs(-) cells (i.e., SK-BR-3 and MDA-MB-231). Taken together, these data confirm a close relationship between the activation of ERα and the E2-induced NGB over-expression in breast cancer. Interesting results are obtained in ERα(-) SK-BR-3 cell line, in which high NGB amount is observed but not under E2 regulation suggesting that an active signaling pathway should exist in this cell line. Consequently, we evaluated the pathways involved. Firstly, we demonstrate that both the impairment of protein degradation and the enhancement of protein synthesis are at the root of E2-dependent rapid NGB up-regulation. Moreover, we showed that both PI3K/PDK/AKT pathway and PKC activation, an upstream activator of AKT, are required for the E2-induced increase of NGB levels in ERα(+) cell lines. In particular, E2 rapidly and persistently activates AKT phosphorylation and the pathway culminates in NGB up-regulation and re-allocation into mitochondria. The high NGB level in whole cells and mitochondria paralleled with the high AKT phosphorylation status found in ERα(-) and Her2/Neu(+) SK-BR-3 cells strongly sustaining the role of AKT. Moreover, no activation of AKT and barely detectable level of NGB are reported in the triple negative MDA-MB-231 cell line. Our results indicate the crucial role of transcription factor CREB (one of the AKT downstream transcriptional factors) in the E2-induced modulation of NGB levels in both MCF-7 and T47D cells. Therefore, the rapid and persistent E2/ERα-induced AKT assure the rapid NGB accumulation into the cells, the long-term transcription of *NGB* gene, and the translocation of NGB into mitochondria where the globin exerts its role in promoting cell survival and avoiding the triggers of apoptotic cascade.

In the second part of this thesis, we tested the effects of different stressors on the level, localization, and function of NGB in wild-type or NGB stable silenced breast cancer cells. The data indicate that hypoxia does not affect NGB protein amount in breast cancer cells,

while nutrient deprivation induces an up-regulation of the globin, suggesting its possible involvement into the autophagic flux. Moreover, NGB level could be considered as a sensor of ROS being up-regulated by ROS (H_2O_2) and by ROS-inducing substances (Pb(IV)), which not re-allocated the globin into mitochondria. In fact, ROS and ROS-inducing compounds rapidly and transiently activate the AKT phosphorylation, unlike E2. Finally, we demonstrated that in MCF-7 cells, an active ERα is at the root of MCF-7 insensitivity to paclitaxel, a chemotherapeutic agent. In the absence of active ERα, paclitaxel significantly reduces the NGB cell content, suggesting that the NGB down-regulation could represent a mechanism by which chemotherapeutic drugs render cancer cells more prone to death.

In the third part of this thesis, we screened different plant-derived polyphenols able to interfere with E2 actions. The selected compounds showed different effects on NGB and E2-induced NGB up-regulation. In particular, we selected Naringenin (Nar) and Resveratrol (Res), which are able to interfere with E2-induced NGB up-regulation; moreover, while Nar does not affect NGB level, Res decreases globin protein amount. Consequently, using ERα(+), ERs(-) and ERs(+) cell lines, we showed different effects of selected compounds on NGB and E2-induced NGB up-regulation, confirming that polyphenols act as E2 agonists and antagonists depending on the receptor content (ERα and ERβ) of specific tissues. Thus, we evaluated the Nar and Res effects on ERα and AKT activation, and demonstrated that Nar effects on decreasing E2-induced NGB levels are mediated by the inhibition of E2-induced AKT phosphorylation. Instead, Res acts through the inactivation of ERα phosphorylation and consequently, decreased AKT phosphorylation due to E2. Finally, Res and Nar, preventing E2-induced NGB up-regulation, enhance paclitaxel effect on inducing apoptosis cell death rendering cancer cell more prone to death.

As a whole, these results show that E2/NGB pro-survival pathway represents a conserved activated mechanism in ERα breast cancer. Thus, we define NGB as a conserved compensatory protein induced by E2 and identify the ERα activated PI3K/AKT signaling as the main intracellular upstream pathway by which E2 affects NGB compartmentalization into mitochondria. Moreover, NGB could be considered as sensor of ROS in breast cancer. Furthermore, our data reveal the pivotal role played by NGB in the E2/ERα-induced anti-apoptotic pathway that abrogates paclitaxel-induced cell death. Finally, data of this thesis indicate that polyphenols, which impairs E2-dependent induction of NGB in breast cancer cells could be useful to reduce cancer cells endurance to chemotherapeutic treatment.

La via di segnale E2/Neuroglobina come bersaglio farmacologico per il cancro correlato all'ormone

RIASSUNTO

Nel 2000, nel sistema nervoso, è stata scoperta la terza eme-globina, chiamata Neuroglobina (NGB). La NGB è una globina esa-coordinata la cui coordinazione è reversibile e controllata dallo stato ossidativo della cellula così come dalla fosforilazione, per cui si pensa che la NGB possa agire come un sensore redox nel sistema nervoso. Lavori sperimentali asseriscono che la NGB è una molecola neuroprotettiva la cui over-espressione potrebbe proteggere il sistema nervoso contro diversi insulti. Questi dati hanno spinto diversi laboratori a cercare composti capaci di aumentare i livelli di NGB. Risultati recenti hanno mostrato che il 17β-estradiolo (E2) induce fortemente l'up-regolazione della NGB del 300% nella linea cellulare di neuroblastoma umano SK-N-BE.

Il 17β-estradiolo (E2) è il più concentrato ed efficiente ormone steroideo sessuale femminile. Nelle donne in premenopausa è prodotto nelle ovaie e i suoi livelli sono significativamente alti durante l'età riproduttiva. E2 controlla molti aspetti della fisiologia umana incluso lo sviluppo, la riproduzione, l'omeostasi e le funzioni cerebrali attraverso l'attività dei suoi recettori, ERα e ERβ. Dato l'ampio ruolo di E2 nella fisiologia umana, non sorprende che esso sia implicato nello sviluppo o nella progressione di numerose malattie, come ad esempio vari tipi di cancro (mammella, ovaio, prostata e endometrio). I principali fattori che influenzano gli effetti di E2 sono lo stato e il bilanciamento dei suoi recettori che risultano modificati nelle cellule di cancro. ERα promuove la proliferazione del cancro alla mammella, ginecologico, e quello delle ghiandole endocrine. Invece, ERβ sopprime la proliferazione delle cellule tumorali e guida verso l'apoptosi. Nel cervello, diversamente da ciò che avviene nei tessuti tumorali extra-nervosi, le funzioni mediate da ERα e da ERβ hanno un fine comune, la neuroprotezione.

Lavori precedenti svolti nel nostro laboratorio indicano che E2, attraverso l'aumento e la riallocazione della NGB nei mitocondri, esercita una funzione anti-apoptotica e protettiva nei neuroni attraverso segnali rapidi e genomici mediati da ERβ. Inoltre, in cellule di cancro alla mammella e epatiche, E2 attraverso ERα induce l'aumento e la riallocazione della NGB nei mitocondri nei quali la globina contrasta la cascata apoptotica indotta dallo stress

ossidativo senza ulteriori effetti sulla proliferazione cellulare indotta da E2, suggerendo un ruolo specifico della NGB nel percorso apoptotico.

Al fine di definire meglio il ruolo funzionale giocato dalla NGB negli effetti cellulari indotti da E2, questa tesi ha come scopi: (i) analizzare le vie di segnale, importanti per la sopravvivenza delle cellule tumorali, che portano all'over-espressione della NGB indotta da E2; (ii) evidenziare la funzione svolta dalla NGB nel cancro correlato con E2 e (iii) selezionare un composto naturale in grado di interferire con il percorso attivato da E2 attraverso ERα, per interferire con l'over-espressione della NGB nelle cellule di cancro alla mammella.

Nella prima parte di questa tesi abbiamo dimostrato che nei tessuti tumorali della mammella umana i livelli di NGB sono elevati, confermando un ruolo chiave della globina nella fisiologia del cancro alla mammella. Inoltre, abbiamo mostrato una forte correlazione tra NGB e ERα. Al fine di ottenere una più precisa correlazione tra i livelli di NGB e l'attivazione di ERα, abbiamo considerato cinque linee cellulari di cancro alla mammella, caratterizzate da una diversa espressione di ERα. La NGB è espressa in tutte le linee cellulari considerate ma l'aumento della globina dipendente da E2 è mantenuta solo nelle linee cellulari ERα(+) (MCF-7, T47D, ZR-75-1), mentre nessuno effetto di E2 è riportato nelle cellule ERs(-) (SK-BR-3 e MDA-MB-231). Tutti questi risultati confermano una stretta relazione tra l'attivazione di ERα e l'over-espressione della NGB indotta da E2 nel cancro alla mammella. Risultati interessanti sono stati ottenuti nella linea cellulare ERs(-) SK-BR-3, in cui sono stati osservati alti livelli di NGB ma non regolati da E2 suggerendo che in questa linea cellulare potrebbe esistere una via di segnale attiva. Quindi abbiamo valutato le vie di segnale coinvolte. Innanzitutto abbiamo dimostrato che sia la riduzione della degradazione della proteina che il miglioramento della sintesi proteica sono alla base della rapida regolazione della NGB dipendente da E2; inoltre sia la via PI3K/PDK/AKT che l'attivazione di PKC, un attivatore a monte di AKT, sono richiesti per l'aumento dei livelli della NGB indotto da E2 nelle linee cellulari ERα(+). In particolare, E2 attiva rapidamente e persistente la fosforilazione di AKT e il percorso culmina nell'aumento dei livelli della NGB e nella traslocazione nei mitocondri. L'elevato livello di NGB in tutta la cellula e nei mitocondri parallelamente all'elevato stato di fosforilazione di AKT trovato nelle cellule ERα(-) e Her2/Neu(+) SK-BR-3 sostengono fortemente il ruolo di AKT. Ulteriore conferma è stata ottenuta nella linea cellulare triplo negativa, MDA-MB-231, che mostra un livello appena rivelabile della NGB e nessuna attivazione di AKT. I nostri risultati indicano il ruolo cruciale del fattore di trascrizione CREB (uno dei fattori trascrizionali a valle di AKT) nella

modulazione dei livelli della NGB indotti da E2 nelle linee cellulari MCF-7 e T47D. Pertanto, la rapida e persistente attivazione di AKT indotta da E2/ERα assicurano il rapido accumulo della NGB nelle cellule, la trascrizione a lungo termine del gene *NGB* e la traslocazione della NGB nei mitocondri, dove la globina esercita il suo ruolo nella promozione della sopravvivenza cellulare e nell'evitare l'innesco della cascata apoptotica.

Nella seconda parte di questa tesi abbiamo testato gli effetti di diversi stressori sul livello, localizzazione e funzione della NGB in cellule di cancro alla mammella. I dati indicano che l'ipossia non influenza la quantità proteica della NGB nelle cellule di cancro alla mammella, mentre la deprivazione dei nutrienti induce un aumento della globina, suggerendo un possibile coinvolgimento della stessa nel flusso autofagico. Inoltre, il livello della NGB potrebbe essere considerato come un sensore di ROS essendo regolato da ROS (H_2O_2) e da sostanze che inducono ROS (Pb (IV)), che non inducono la traslocazione della NGB nei mitocondri. Infatti, i ROS e i composti che inducono ROS attivano rapidamente e temporaneamente la fosforilazione di AKT, diversamente da E2. Infine, abbiamo dimostrato che nelle cellule MCF-7, un ERα attivo è alla base dell'insensibilità delle MCF-7 al paclitaxel, un agente chemioterapico. In assenza di ERα attivo, il paclitaxel riduce in modo significativo i livelli della NGB, suggerendo che la riduzione della globina potrebbe rappresentare un meccanismo mediante il quale i chemioterapici possano rendere le cellule tumorali più inclini alla morte.

Nella terza parte di questa tesi abbiamo analizzato vari polifenoli derivanti da piante in grado di interferire con le azioni di E2. I composti selezionati hanno mostrato diversi effetti sulla NGB e sull'aumento della NGB indotta da E2. In particolare, abbiamo selezionato Naringenina (Nar) e Resveratrolo (Res), che sono in grado di interferire con l'aumento della NGB indotta da E2; inoltre, mentre Nar non influisce sui livelli della NGB, Res diminuisce la quantità proteica della globina. Di conseguenza, utilizzando linee cellulari ERα(+), ERs(-) e ERs(+), abbiamo mostrato diversi effetti dei composti selezionati sulla NGB e su l'aumento della NGB indotta da E2, confermando che i polifenoli agiscono come agonisti e antagonisti di E2 a seconda del contenuto dei recettori (ERα e ERβ) in specifici tessuti. Conseguentemente, abbiamo valutato gli effetti di Nar e Res sull'attivazione di ERα e AKT e abbiamo dimostrato che gli effetti di Nar sulla riduzione dei livelli di NGB indotti da E2 sono mediati dall'inibizione della fosforilazione AKT indotta da E2. Invece, il Res agisce attraverso l'inattivazione della fosforilazione di ERα e, conseguentemente, diminuendo la fosforilazione di AKT indotta da E2. Infine, abbiamo dimostrato che sia il Res che la Nar, prevenendo l'aumento della NGB indotta da E2, aumentano l'effetto del paclitaxel

sull'induzione della morte cellulare apoptotica rendendo le cellule cancerose più inclini alla morte.

Nel complesso, questi risultati mostrano che la via di segnale di sopravvivenza E2/NGB rappresenta un meccanismo attivato conservato nel tumore alla mammella ERα(+). Quindi, abbiamo definito la NGB come una proteina compensatoria conservata indotta da E2 e abbiamo identificato la via di segnale PI3K/AKT attivata da ERα come la principale via intracellulare a monte con cui E2 influenza la compartimentalizzazione della NGB nei mitocondri. Inoltre, la NGB potrebbe essere considerata come un sensore di ROS nel cancro alla mammella. I nostri dati rivelano il ruolo fondamentale svolto dalla NGB nel percorso anti-apoptotico indotto da E2/ERα che impedisce la morte cellulare indotta da paclitaxel. Infine, i dati di questa tesi indicano che i polifenoli, che compromettono l'induzione della NGB indotta da E2 nelle cellule di cancro alla mammella, potrebbero essere utili per ridurre la resistenza delle cellule tumorali al trattamento chemioterapico.

1. INTRODUCTION

1.1 Neuroglobin

Haemoglobin (Hb) and myoglobin (Mb) are probably the best-characterized heme-proteins. The heterotetrameric Hb transports oxygen in the blood, whereas the monomeric Mb facilitates the diffusion of oxygen to the mitochondria of muscle cells (Gödecke et al., 1999). For many decades, it was assumed that these two proteins represented the only two heme-globins in the human body. It was so until 2000 when Burmester and colleagues discovered in the nervous system the third heme-globin in human and mouse, for this reason they called it Neuroglobin (NGB) (Burmester et al., 2000). Since then, NGB has been identified in various mammalian, avian, reptilian, amphibian and fish species (Burmester et al., 2004). NGB displays a homology degree of 94% between mouse and human (Burmester et al., 2004) and results close to invertebrates nervous globins (Fago et al., 2004), whereas its homology with Hb and Mb is very different (Ascenzi et al., 2014).

The human NGB gene, located on chromosome 14q24, has a unique exon-intron structure. NGB shares Mb typical structure: it is a monomer of about 150 amino acids with a molecular weight of 17 kDa (Burmester et al., 2000; Dewilde et al., 2001) and the typical 3-over-3 α-helical sandwich structure (Fig. 1.1) (Ascenzi and Brunori, 2016).

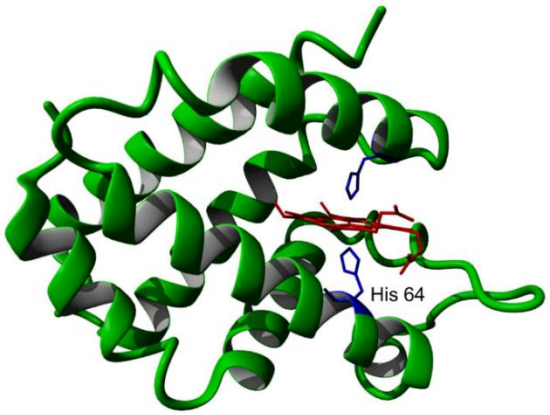

Figure 1.1 Crystal structure of human NGB showing the heme group (red) and iron bound histidine side chains (blue) (Brittain, 2012).

In absence of an external ligand, there are significant differences between Hb or Mb and NGB. Hb and Mb are so-called penta-coordinate globins, in which the iron (Fe^{2+}) is bound by four nitrogen atoms of the porphyrin ring and the proximal histidine in the F helix (HisF8). Upon oxygenation, O_2 binds to the free sixth coordination site of the Fe^{2+}. NGB is a hexa-coordinate globin: in the deoxy state, the distal histidine in the E helix (HisE7) is bound to the sixth coordination position of the Fe^{2+}. Any external ligand has therefore to compete with HisE7 for the binding of Fe^{2+} (Hankeln et al., 2005; Trent and Hargrove, 2002).

1.2 Neuroglobin function

During the past decade, the peculiarity of NGB solicited several researchers to detect its functions. NGB comes up as a hexa-coordinated globin, but its coordination is reversible and controlled by the oxidative state of the cell, as well as by phosphorylation (Hamdane et al., 2003; Jayaraman et al., 2011). For this reason, NGB has been postulated to act as a redox sensor (Ascenzi et al., 2014; Watanabe et al., 2012). As other globins, NGB reversibly binds gaseous ligands, like O_2, NO and CO, displays pseudo-enzymatic properties, among which the detoxification of RNS and ROS species, facilitates the O_2 supply to mitochondria of metabolically active neurons and retinal rod cells, and catalyses the conversion of nitrite to NO under hypoxic conditions (Ascenzi et al., 2014; Brunori et al., 2005). Remarkably, NGB reactivity is facilitated allosterically by phosphorylation and 14-3-3 binding (Jayaraman et al., 2011). Another role recently attributed to NGB is the protection against apoptosis by binding to the Gα protein to inhibit GDP dissociation (Greenberg et al., 2008). Moreover, the interaction between NGB and cytochrome c1 has been reported in mitochondria of primary mouse cortical neurons by confocal microscopy and co-immunoprecipitation assay (Yu et al., 2016).

Several studies have established a close link between NGB and mitochondria. Ferrous NGB reacts very rapidly with cytochrome *c*(III), *via* the formation of an intermediate complex, followed by fast electron transfer (Bonding et al., 2008; Fago et al., 2006). Cytochrome *c*, a heme-protein loosely associated with the inner membrane of the mitochondrion, is an essential component of the electron transport chain, where it transfers electrons between complexes III and IV. Different analyses, including NMR analysis, surface plasmon resonance studies, protein modified quartz nanopipettes, identify the formation of a weak complex between NGB and cytochrome *c* (Ascenzi et al., 2016). Moreover, confocal microscopy and immunoprecipitation assays in human neuroblastoma cells (De Marinis et al., 2013b) and in

cancer cells (Fiocchetti et al., 2015; Fiocchetti et al., 2014) demonstrated that the cytochrome *c*-NGB association requires high levels of NGB and its translocation into the mitochondria. Both processes are induced by 17β-estradiol (E2) but not by oxidative stress inducers.

Other works demonstrated an interaction between NGB and VDAC, a voltage dependent anion channel indispensable for the control of mitochondria permeability transition pore opening, through co-immune-precipitation and immunocytochemistry studies in primary cultures of mouse cortical neurons (Yu et al., 2013a; Yu et al., 2012b; Yu et al., 2013b). In addition, bioinformatics investigations identified the possible interaction interfaces between NGB and VDAC molecule that, however, is usually buried within the mitochondrial outer membrane (Guidolin et al., 2014). Although less energetically favoured (and likely unstable), an alternative suggested configuration involved the binding of NGB to residues located at the boundary of the pore formed by the VDAC molecule (Guidolin et al., 2014). This alternative configuration opens the possibility for NGB to modulate directly the permeability of the outer mitochondrial membrane (Bayrhuber et al., 2008), which represents the key event leading to apoptotic cell death (Tait and Green, 2010).

Other reports show that ferrous NGB, present in normoxia, is converted to the ferric form during oxidative stress so that it associates with the lipid raft micro-domain-associated protein flotillin-1 and with the α subunit of a protein G responsible for inhibition of adenylate cyclase. This association avoids protein G activation and impairs the decrease of the cAMP level, which is typical of oxidative conditions, and the apoptotic cell death (Watanabe et al., 2012).

Experimental works show that NGB is a neuroprotective molecule which overexpression could protect cultured neurons and the animal nervous system against several insults (Greenberg et al., 2008; Sun et al., 2001; Yu et al., 2012a). In fact, several injuring stimuli, such as hypoxia (Brunori et al., 2005), H_2O_2 (De Marinis et al., 2013b), and lipopolysaccharide (De Marinis et al., 2013a) slightly induce NGB expression. As a whole, these reports lead to consider NGB as a stress-inducible protein which up-regulation results in neuroprotection (Fiocchetti et al., 2013). This assumption prompted several laboratories to find compounds able to increase NGB expression levels bypassing the limit of the protein to cross plasma membrane (Watanabe and Wakasugi, 2008). Cobalt, deferoxamine, hemin, short chain fatty acids (i.e. valproic and cinnamic acids), HIF1α, and VEGF were reported to enhance NGB levels, but the high concentrations of some of these compounds to obtain the effect generate several concerns on their possible application due to the possible presence of

serious side effects (Haines et al., 2012; Jin et al., 2012; Jin et al., 2011). Recent data showed that in human neuroblastoma cell line (SK-N-BE), in mouse primary hippocampal neurons, and in primary astrocytes (De Marinis et al., 2010; De Marinis et al., 2011), NGB is strongly up-regulated (300%) by the steroid hormone: 17β-estradiol (E2).

1.3 Estrogens

Estrogens, like other steroid hormones, present a cyclopentanophenanthrene structure whose synthesis begins from cholesterol. Estrogens exhibit a broad spectrum of physiological functions ranging from regulation of ovary cycle, bone density, brain function and cholesterol homeostasis (Ascenzi et al., 2006; Chen et al., 2008). Both in men and women, estrogens are present but women show highest estrogen levels at the reproductive age. These hormones are principally produced in the ovary and adrenal cortex. Women naturally produce three estrogens (Ascenzi et al., 2006; Watson et al., 2008): 17β-estradiol (E2), estrone (E1) and estriol (E3). E2 is the most active form of estrogens: it possesses the strongest affinity for its receptors and it is produced in the largest quantity, raising 0.73-1.47 nM during the ovulatory step of the menstrual cycle. After menopause, plasma estradiol levels drop to less than 0.073 nM because ovary ceases to produce it. E1 is a less effective metabolite of estradiol and it is produced mainly in the liver as well as in the adrenal gland and adipose tissue deriving from androstenedione. Unlike E2, E1 levels do not change in post-menopausal women because the adrenal gland still produce its precursor androstenedione. E3, the third endogenous estrogen, is a metabolite of estradiol. E3 is the principal estrogen produced by the placenta during pregnancy (Ascenzi and Brunori, 2016) while its circulating levels are lower than estradiol and E1 in non-pregnant women (Ascenzi et al., 2006; Liang and Shang, 2013; Ruggiero and Likis, 2002).

The biosynthesis of E2 requires cytochrome P450 aromatase enzyme, which is present in ovary, placenta, testis, prostate, adipose tissue, liver, bone, breast, muscle, endothelial cells and brain. In premenopausal women, E2 synthesis is mainly realized by ovaries, that represents the main sources of circulating E2, while in post-menopausal women and in men its production is accomplished by the local conversion of testosterone and androstenedione to E2 by aromatase enzyme in extra gonadal sites (Simpson, 2003).

Despite the classical idea concerning E2 as the female hormone, its actions are important also for men's physiology (Nilsson et al., 2001; Smith et al., 2008; Zirilli et al., 2008).

1.4 Estrogen receptors

The biological actions of estrogens are principally mediated by two specific estrogen receptors (ERs), ERα and ERβ, which belong to the nuclear receptor (NR) superfamily, a family of ligand-regulated transcription factors (Ascenzi et al., 2006; Gronemeyer et al., 2004; Heldring et al., 2007). ERα and ERβ are encoded by two different genes (ESR1 and ESR2, respectively) expressed on two distinct chromosomes (locus 6q25.1 and locus 14q23-24.1, respectively) (Ascenzi et al., 2006; Boggess et al., 2006; Enmark et al., 1997; Gosden et al., 1986; Kraus et al., 2002; Krust et al., 1986; Luisi et al., 2006). They are composed of five independent but interacting functional domains: A/B, C, D, E and F. The A/B or NH2-terminal region represents the most variable domain in both sequence and length and contain a transactivation domain (AF1) with ligand-independent function and a co-regulatory domain that is responsible for the recruitment of co-activators and co-repressors. The C or DNA-binding domain binds estrogen responsive elements (EREs) present on promoters of E2-responsive genes. The carboxyl-terminal regions or E and F containing the ligand binding domain (LBD) and have a ligand-dependent transactivation function (AF2). This region is also responsible for the binding to co-regulatory and chaperone proteins, as well as for receptor dimerization and nuclear translocation (NLS). Finally, the D region contains several functional domains, including the hinge domain, part of the ligand-dependent activating domain and the nuclear localization signal (Fig. 1.2) (Ascenzi et al., 2006; Nilsson et al., 2001).

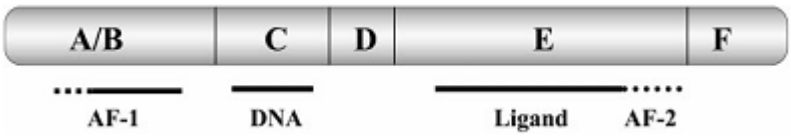

Figure 1.2 Diagrammatic representation of the domain structure of nuclear receptors (Nilsson et al., 2001).

1.4.1 Estrogen receptors action mechanisms

ERs are localized in different part of the cell. The non-activated forms are mainly located in the cytoplasm associated with Heath Shock Proteins (e.g., HSP90 and HSP70). E2 binding impairs this association, ERs dimerize and migrate to the nucleus, where they recruit different chromatin re-modeler or transcriptional co-regulators and bind directly to the ERE sequence on DNA (Marino et al., 2006; Nalvarte et al., 2010; Nilsson and Gustafsson, 2011).

Moreover, the ER/E2 complex could interact with other transcriptional factors (e.g., AP-1 and Sp-1) to indirectly enhance gene transcription (Acevedo and Kraus, 2004; Paech et al., 1997; Weihua et al., 2003).

The genomic action of E2 is realized at least 2 h after E2 stimulus and is responsible for many of E2 described functions (Farach-Carson and Davis, 2003; Marino et al., 2005). In addition to the well-studied transcriptional effects of E2, there are rapid effects occurring within seconds or minutes after addition E2 (Simoncini et al., 2004; Song and Santen, 2006; Song et al., 2005; Warner and Gustafsson, 2006; Wong et al., 2002). Various signalling pathways are activated upon E2 binding ERs. These rapid events may be classified into four main signalling cascade: phospholipase C (PLC)/protein kinase C (PKCs), Ras/Raf/MAPK, phosphatidyl inositol 3 kinase (PI3K)/AKT (Acconcia and Marino, 2011; Adlanmerini et al., 2014), and cAMP/protein kinase A (PKA) (Chen et al., 1998; Farhat et al., 1996; Gu and Moss, 1996; Malyala et al., 2005; Marino et al., 2006; Picotto et al., 1996; Picotto et al., 1999). The activation of these rapid pathways, due to E2, is guaranteed by the localization of ERs at the plasma membrane micro-domains like lipid rafts and caveolae (Levin, 2009; Marino and Ascenzi, 2008; Marino et al., 2006; Márquez et al., 2006; Song et al., 2005). Remarkably, ERα and ERβ can be localized at the plasma membrane through the direct interaction with caveolin-1 to initiate the signal transduction pathway(s) (Acconcia et al., 2005a; Galluzzo et al., 2007; Roepke et al., 2011). Indeed, ERs require a post translational modification by lipid (i.e. S-palmitoylation) to be localized at the plasma membrane, to interact with caveolin-1, and to initiate the E2 rapid signal cascade activation (Acconcia et al., 2005a; Galluzzo et al., 2007; Marino and Ascenzi, 2008; Pedram et al., 2007).

To complicate this picture, in 1997, a G protein-coupled receptor (GPR30 or GPER) was cloned and established as another E2-binding membrane receptor (Carmeci et al., 1997), but experiments carried out in cells from ERα/ERβ KO mice show no response to E2, despite the presence of GPER (Pedram et al., 2006). Thus, a role for GPER as an ER remains controversial. Therefore, there is support that GPER can collaborate in the setting of E2/membrane ERα signalling (Levin, 2009). Similar to ERs, GPER is expressed throughout the organism.

1.4.2 ER-mediated E2 functions

ERα receptor is principally expressed in the uterus, liver, breast, kidney and heart, while ERβ in the ovary, prostate, gastrointestinal tract, bladder and hematopoietic and central nervous system. Both ERα and ERβ are expressed in the mammary gland, epididymis, thyroid, adrenal, bone and some brain areas (Matthews and Gustafsson, 2003).

ERβ appears to act as a dominant regulator in E2 signalling, and when co-expressed with ERα it causes a concentration-dependent reduction of ERα-mediated transcriptional activation (Matthews and Gustafsson, 2003) and the repression of ERα-mediated effects (Marino et al., 2006). The relative levels of ERα and ERβ define the different biological response to estrogens in particular regarding proliferation, survival and apoptosis.

ERα activation mediates the proliferative and anti-apoptotic estrogen effects, while ERβ seems to oppose ERα actions on cell proliferation by modulating the expression of many ERα-regulated genes and exhibits pro-apoptotic and pro-differentiation properties (Acconcia and Marino, 2011; Fox et al., 2009; Marino et al., 2006; Thomas and Gustafsson, 2011). Moreover, ERα and ERβ opposite function on cellular response may arise from differences between the receptors rapid signalling pathway activated. ERα is the primary endogenous mediator of rapid E2 actions. The ERα membrane initiated signals are critical for DNA synthesis and cell cycle progression. Accumulating evidence identifies at least two conserved pathways in membrane ERα-based E2 rapid signalling. The E2-dependent activation of the ERK component of the MAPK family and the AKT kinase in the PI3K axis occur in a variety of cell lines. Nevertheless, the ability of the E2/ERβ complex to activate rapid signals is less known. The E2-dependent activation of the p38 component of the MAPK family has been correlated to the ERβ-mediated function (Acconcia et al., 2005b; De Marinis et al., 2010; Galluzzo et al., 2007; Galluzzo et al., 2009). Beyond the contrasting outcomes of ERα and ERβ activities in mediating E2 regulation of cell survival and apoptosis, the receptors activities result synergic in the cardio-protective and the neuroprotective E2 effects (Vitale et al., 2009).

1.5 E2 and cancer

Given the widespread role of estrogen in human physiology, it is not surprising that estrogens are also involved in the development or progression of numerous chronic disease,

which include, but are not limited to, various type of cancer (breast, ovarian, colorectal, prostate and endometrial), osteoporosis, neurodegenerative and cardiovascular disease, obesity (Deroo and Korach, 2006).

Estrogen and its receptors are involved in the development of many types of malignant tumors, which are generally classified in four groups: breast and gynaecologic cancers (cervical endometrial, ovarian), endocrine gland cancers (adrenocortical, ovarian, pancreatic, and thyroid), digestive cancers (colorectal, esophagael, liver and pancreatic), and lung carcinoma (Chen et al., 2008). Most of these tumors can express both ERα and ERβ. ERα appears to promote the proliferation of breast, gynecologic and endocrine gland cancers. In contrast to ERα, ERβ suppresses the proliferation of tumor cells. The alteration of both ERs expression is an important step in the development and progression of hormone-related cancers, and it influences cancer response to therapy (Foley et al., 2000; Leav et al., 2001; Musgrove and Sutherland, 2009; Rutherford et al., 2000; Thomas and Gustafsson, 2011; Zhu et al., 2004).

Different studies show that when a normal breast tissues become tumorigenic, the amount of ERα RNA increases whereas the amount of ERβ decreases (Leygue et al., 1998). Over-expression of ERβ prevents establishment and growth of breast tumors in a subcutaneous xenograft mouse model (Behrens et al., 2007). Restoration of ERβ in ovarian cancer cells results in enhancement of apoptosis of tumor cells, and a strong inhibition of their proliferation and invasion (Lazennec, 2006). E2-mediated proliferation of endometrial cells is blocked by transfection with ERα antisense DNA (Taylor et al., 2002; Zhang et al., 2006), but not with the ERβ antisense DNA (Zhang et al., 2006).

The estrogen involvement in cancer progression is complicated by the multiple function of estrogen in cells, which can be either adverse or beneficial in cell type and organ dependent manner.

1.6 NGB, E2 and cancer

Although NGB was firstly identified in the nervous system, Emara and colleagues showed that NGB is expressed in several nervous and non-nervous cancer and in their normal counterparts (Emara et al., 2010). On the other hand, Gorr and colleagues reported that no significant levels of NGB transcript could be detected in non-nervous tumors and normal organs (Gorr et al., 2011). In addition, a role as tumor suppressor has been reported in NGB-

overexpressing hepatocarcinoma cells where the globin acts as a linkage between O_2/ROS signals and intracellular signalling that is important for cell proliferation (Zhang et al., 2013a; Zhang et al., 2013b). All together, these results highlight that NGB role in cancer is unclear. In our laboratory, we demonstrated that NGB is an E2-inducible protein in both neurons and non-nervous cancer cells (Fiocchetti et al., 2014; Nuzzo et al., 2014). The cancer biological responses to E2 can be either adverse or beneficial; in fact, they are related to the state and the balance of its receptors ERα and ERβ, which mediate the pro-survival and pro-apoptotic E2 actions, respectively (Acconcia and Marino, 2011; Acconcia et al., 2005b; Nuzzo et al., 2014). In hepatocarcinoma (HepG2) and in breast cancer cells (MCF-7) E2 induced the up-regulation of NGB in a dose- and time-dependent manner. Moreover, E2 stimulation modified NGB intracellular localization, inducing a significant reduction of NGB in the nucleus with a parallel increase of NGB in the mitochondrial compartment in which the globin counteracts apoptosis induced by oxidative stress without any further effect on E2-induced cell proliferation, suggesting a specific role of the globin in the apoptotic pathway (Fiocchetti et al., 2014). E2 effects in promoting cancer progression are typical of ERα expressing cells, whereas the hormone acts as a pro-apoptotic agent in ERβ-expressing cancer cells (Acconcia and Marino, 2011). Surprisingly, also in colon derived ERβ-expressing cancer cells (DLD-1), where the pro-apoptotic function of the hormone is well known (Galluzzo et al., 2007; Marino et al., 2006), E2 up-regulates and re-allocates NGB into mitochondria. However, E2-induced over-expression of NGB in DLD-1 cells does not interfere with the pro-apoptotic function of E2 in resting condition. On the contrary, only in a deeply oxidizing cellular status, E2-induced NGB, re-allocated in mitochondria, affords to impair the cytosolic release of cytochrome *c* by interacting with it and consequently preventing the apoptotic cascade (Fiocchetti et al., 2015).

2. AIMS

In the last few years, the interest about the role of NGB in cancer has grown because the possibility that the globin may be similarly modulated and operate through the same pathways that occur in nervous cells. As discussed in the previously chapter, the role of NGB in cancers is not still clear (Emara et al., 2009; Gorr et al., 2011).

Previous work performed in our laboratory indicates that E2 *via* ERβ genomic and rapid signals exerts an anti-apoptotic and protective function in neurons by up-regulating and re-allocating NGB into mitochondria (De Marinis et al., 2010; De Marinis et al., 2013b; Fiocchetti et al., 2012; Fiocchetti et al., 2013). In addition, in hepatocarcinoma and in breast cancer cells, E2 *via* ERα induced the up-regulation and re-localization of NGB into mitochondrial compartment in which the globin counteracts apoptosis induced by oxidative stress without any further effect on E2-induced cell proliferation, suggesting a specific role of the globin in the apoptotic pathway (Fiocchetti et al., 2014).

As a whole, these data raise the hypothesis that NGB could represent one of the vital mechanisms triggered by E2 to increase the survival of cancer cells in the presence of oxidative stress. Thus, the aim of this thesis is investigate functions and action mechanisms of NGB as an E2-induced compensatory protein in E2-related breast cancer in order to develop a new therapeutic strategy against E2-related breast cancer.

To reach this aim, the main goals of this thesis are:

- To dissect the signalling pathways, important for cancer cell survival, which up-stream E2-induced NGB over-expression.
- To evidence the function played by NGB in E2-related cancer.
- To screen molecules able to interfere with E2-activated pathway *via* ERα, in order to selectively interfere with NGB over-expression in breast cancer cells.

3. MECHANISMS AT THE ROOT OF E2-INDUCED NGB UP-REGULATION IN BREAST CANCER

3.1 Introduction

Breast cancer, a leading tumor in women, is the second most common cancer in the world (1.67 million new cancer cases in 2012) (Ferlay et al., 2015) and despite important advances in research, it remains a major health problem and represents a top biomedical research priority. Originally, the classification of breast cancers was founded on their histopathological type and grade; more recently, this classification has been refined including the expression of at least three cancer biomarkers that are used to predict prognosis and to define the therapeutic treatments (Vuong et al., 2014). The majority of breast cancers are defined as E2 responsive based on the nuclear expression of the estrogen receptor α subtype (ERα positive) (Acconcia and Marino, 2011; Au et al., 2007; Boggess et al., 2006; Taylor et al., 2002; Zhang et al., 2006). In addition, progesterone receptor (PR) expression, regulated by E2, indicates a functioning ERα signaling pathway (Vuong et al., 2014). The ERα expression predicts the potential response of breast cancer to the endocrine therapy (i.e., tamoxifen, fulvestrant, aromatase inhibitors) (Mohibi et al., 2011; Vuong et al., 2014); on the other hand, the over-expression of human epidermal growth factor receptor 2 (Her2/Neu) is considered as an indicator of a poor prognosis (Rakha et al., 2010; Valentin et al., 2012; Vici et al., 2015; Vuong et al., 2014). Although the inclusion of these three breast cancer biomarkers helped to partially solve the therapeutic approach to the heterogeneity of this cancer, the existence of the endocrine therapy resistance in ERα positive breast cancer (Bruce et al., 2014; Murphy et al., 2011) further complicate this scenario. A step forward in the improvement of ERα positive breast tumor treatment could derive from the knowledge of the E2/ERα molecular mechanisms that drive cancer cells to the survival and stress adaptation.

A good candidate that could represent a new, complementary and/or alternative target to ERα is NGB. Since its discovery, it has been assessed that overexpressed NGB has cytoprotective properties in neurons, including anti-apoptotic function, NOS and ROS scavenging, and signaling protein against hypoxic/ischemic and oxidative stresses (Ascenzi et al., 2016). Experimental data indicate that, until now, the correlation between NGB expression and non-nervous cancer progression is still uncertain and openly debated (Emara

et al., 2009; Oleksiewicz et al., 2011; Zhang et al., 2013b). Recently we show that, as well in neurons, NGB is up-regulated and reallocated in mitochondria by E2 in several E2-dependent cancer cells (breast, hepatoma, and colon cancer cells) acting as a pro-survival, anti-apoptotic protein (Fiocchetti et al., 2015; Fiocchetti et al., 2014). In particular in MCF-7 cells, E2 induces a time- and dose-dependent increase of NGB protein levels acting *via* both ERα extra-nuclear signals and transcriptional events, although in NGB promoter gene any canonical Estrogen Responsive Elements (ERE) exists (Guglielmotto et al., 2016).

Here, we hypothesize that E2/NGB pathway could represent a common activated process which takes part to the defense mechanisms established by ERs positive breast cancer cells to adapt themselves and to counteract the micro-environmental stress condition. By using different ERs positive and negative breast cancer cell lines, the molecular pathway which lead to the E2-dependen NGB over-expression, its re-allocation to the mitochondria, and to the anti-apoptotic activity of this globin have been assessed.

3.2 Results

3.2.1 Correlation between NGB and ERα in breast cancer tissues

As described in the first chapter, the correlation between NGB levels and cancer is still unclear (Emara et al., 2010; Gorr et al., 2011). Thus, we first evaluate the NGB levels in breast cancer specimens kindly provided by Divisione Senologica, Ospedale Belcolle, Viterbo, (Prot.N. 2012/CE Lazio 1). Fifteen ERα positive ductal carcinoma specimens (T=tumor) were selected from 55-75 years old patients. Samples of non-proliferative ductal cells of the same patients were used as control (N=normal). In these tissues the levels of NGB and ERα were determined by Western Blot. In addition, the activation of ERα by following its phosphorylation status (pERα) and the level of Bcl-2, progesterone receptors (PRA and PRB) which gene expressions are regulated by E2/ERα transcriptional pathways were evaluated. In addition, the epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (Her2/Neu) expression has been evaluated. Fig. 3.1 shows the typical Western Blot of three specimens (# 5, # 10, # 13). Although with different level of expression, all considered breast tumor samples show the amplification of the Her2/Neu receptor and the higher expression of ERα relative to the paired normal-tissues sample. Interestingly, a large amount of ERα in tumor samples is phosphorylated in Ser118 indicating the activated status of the receptors (Fig. 3.1 A) as further confirmed by the analysis of E2-responsive Bcl-2 protein

which is overexpressed in cancer specimens respect to normal counterpart (Fig. 3.1 C). Furthermore, the higher expression of PRA and B are shown in #5 tumor specimen respect to #10 and #13 tumor samples that show only barely detectable levels (Fig. 3.1 B). Intriguingly, none of the reported receptors are expressed at relevant amount in normal tissues counterpart (Figs. 3.1 A and B). Conversely, EGF receptor is highly expressed in the normal breast tissues than in the respective tumor tissue samples (Fig. 3.1 B). Finally, NGB expression has been detected only in tumoral samples versus their matched normal counterpart confirming a key role of the globin in breast cancer physiology (Fig. 3.1 C).

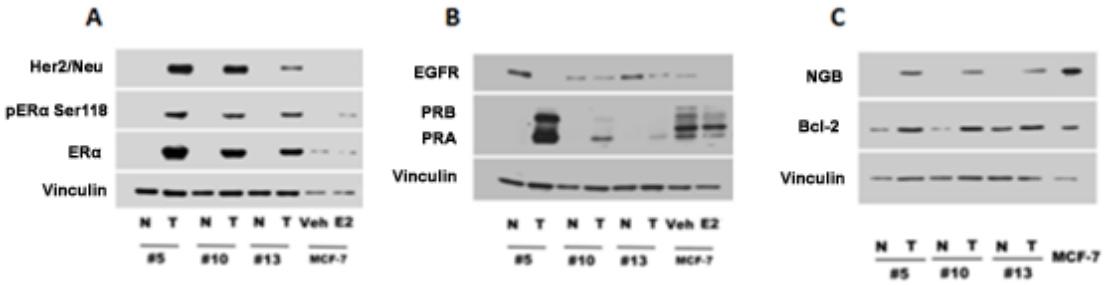

Figure 3.1 Analysis of (A) Her2/Neu, ERα, pERα Ser118, (B) EGFR, PRB, PRA, (C) NGB and Bcl-2 in three different ductal carcinoma samples from post-menopausal patients (#5, #10, #13, N=Normal and T=tumor). The amount of protein was normalized in comparison with vinculin protein levels.

Table 3.1 shows the significant Pearson's coefficient between NGB and ERα levels in tumoral breast cancer tissues. Moreover a strong correlation between NGB and Bcl-2 is observed. On the other hand, no significant correlation between NGB and either PRA, PRB, Her2/Neu or EGFR was evidenced (data not shown).

Correlation	NGB	ERα	pERα	Bcl-2
NGB	1.00	0.96**	0.97**	0.98**
ERα	0.97**	1.00	0.99***	0.89*
pERα	0.97**	0.99***	1.00	0.86*
Bcl-2	0.98**	0.89*	0.86*	1.00

Table 3.1 Pearson's correlation coefficient across normal breast and breast cancer tissues of patients (n = 15). $P > 0.05$ was calculated by two-tailed test. *, moderate positive correlation; **, strong positive correlation; ***, very strong positive correlation.

As a whole, these data sustain the strong correlation between NGB, ductal cancer and ERα activation.

3.2.2 Correlation between NGB levels and ERα in breast cancer cells

In order to obtain a more precise correlation between NGB level and ERα activation, five breast cancer cell lines, characterized by a different ERα level, were considered. In particular, the ERα positive MCF-7, ZR-75-1, and T47D as well as the ERα negative MDA-MB-231 and SK-BR-3 breast cell lines were chosen. As shown in Figs. 3.2 A and B, MCF-7 cells show the highest level of ERα followed by ZR-75-1 and T47D cell lines, whereas MDA-MB-231 and SK-BR-3 cells are ERα negative.

As previously reported (Totta et al., 2014), 24 h of E2 treatment (10 nM) significantly reduces the ERα levels in all the ERα positive cell selected; however, the amount of receptor is still detectable and higher in MCF-7 with respect to ZR-75-1 and T47D cells (Fig. 3.2 B). NGB levels are barely detectable in un-stimulated ERα positive breast cancer cells (Fig. 3.2 C, vehicle). E2 stimulation significantly increases NGB levels, which is higher in MCF-7 with respect to ZR-75-1, and T47D cells (Fig. 3.2 C).

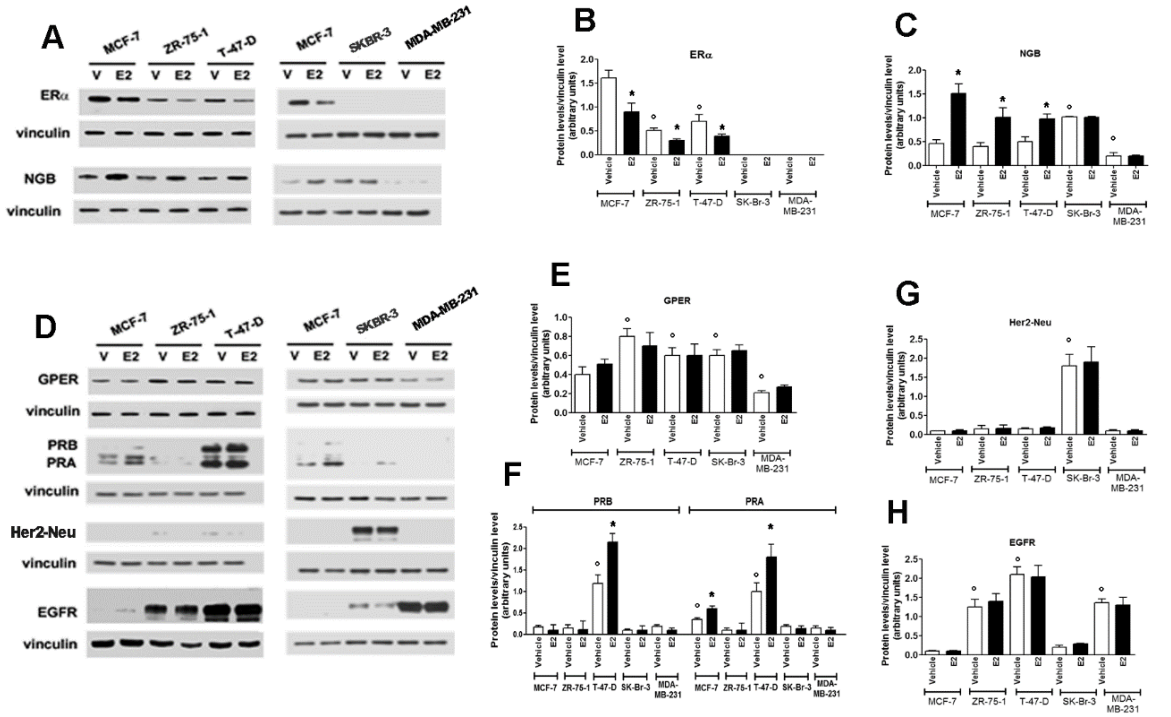

Figure 3.2 Expression of hormone /growth factor receptors and NGB in breast cancer cell. (A) Western blot analysis of ERα and NGB protein levels was performed in MCF-7, ZR-75-1, T47D, MDA-MB-231 and SK-BR-3 cell lines treated with either vehicle or E2 (10 nM; 24h). **(D)** Western blot analysis of GPER, PRB, PRA, Her2/Neu, EGFR protein levels was performed in MCF-7, ZR-75-1, T47D, MDA-MB-231 and SK-BR-3 cell lines treated with either vehicle or E2 (10 nM). The amount of protein was normalized in comparison with vinculin protein levels. Densitometric analysis are reported in: **(B)** ERα, **(C)** NGB, **(E)** GPER, **(G)** Her2/Neu, **(F)** PRB and PRA, **(H)** EGFR, in all of cancer cells considered. Data are means ± S.D. of three different experiments. P<0.001 was determined with ANOVA test versus vehicle (*) and versus vehicle of MCF-7 (°).

The Pearson's coefficient (Table 3.2) shows the strong positive correlation between NGB and ERα levels in E2-stimulated breast cancer cells.

Correlation	NGB	ERα	GPER	PRA	PRB	EGFR	Her2/Neu
NGB	1.00	0.96**	0.08	0.91*	0.15	-0.31	-0.34
ERα	0.96**	1.00	-0.07	0.98**	0.26	-0.08	-0.30
GPER	0.08	-0.07	1.00	-0.16	0.30	0.16	0.30
PRA	0.91*	0.98**	-0.16	1.00	0.31	-0.31	-0.33
PRB	0.15	0.26	0.30	0.31	1.00	0.01	0.07
EGFR	-0.31	-0.08	0.16	-0.31	0.01	1.00	0.14
Her2/Neu	-0.34	-0.30	0.30	-0.33	0.07	0.14	1.00

Table 3.2 Pearson's correlation coefficient across all cancer cells stimulated with 17β-estradiol (n = 50). P> 0.05 was calculated by two-tailed test. *, moderate positive correlation; **, strong positive correlation.

Surprisingly, data reported in Fig. 3.2 C indicate that an E2-independent NGB up-regulation may occur. Indeed, the ERα negative SK-BR-3, but not MDA-MB-231 (triple negative breast cancer cells) (Chavez et al., 2010), contain high amount of NGB, which is not under E2 regulation. This unexpected result prompted us to evaluate if a positive or negative correlation exists between the levels of NGB and other receptors considered as biomarkers of breast cancer. First of all the levels of the other ER subtype (i.e., ERβ) and of the novel G-protein estrogen receptor (GPER) (Maggiolini and Picard, 2010; Prossnitz et al., 2008) has been assessed. As expected (Huang et al., 2014), the ERβ subtypes expression is barely detectable in breast cancer cells (data not shown), whereas GPER is expressed in all cell line considered, showing the lowest level in MDA-MB-231 cells (Fig. 3.2 E). In addition, higher amount of PRA and PRB are detected in T47D than in MCF-7 cells, while, these receptors are completely absent in ZR-75-1 cells (Fig. 3.2 F). E2 (10 nM, 24 h) treatment significantly increases PRs levels in ERα and PR positive cells (i.e., MCF-7, and T47D cells), while it does not change GPER levels in all considered cancer cell lines (Fig. 3.2 E). Table 3.2 confirms that a strong positive correlation exists between PRA and ERα and evidences a moderate positive correlation between NGB levels and PRA levels in E2-stimulated breast cancer cells. These results sustain that only ERα and ERα-dependent receptors (i.e., PRA) are involved in E2-induced NGB up-regulation. No significant correlation is found between NGB levels and the levels of GPER or Her2/Neu (Fig. 3.2 G and Table 3.2) or EGFR (Fig. 3.2 H and Table 3.2).

3.2.3 E2-induced NGB up-regulation does not require the direct NGB gene transcription

Although data reported in Table 3.2 indicate that an active ERα is required for E2-induced NGB up-regulation, the NGB promoter does not contain any ERE (Cutrupi et al., 2014; Guglielmotto et al., 2016). Thus, the E2-induced NGB protein accumulation could be dependent by the regulation of protein degradation. Fig. 3.3 A shows that both the proteasomal inhibitor (MG-132, 1 μM, 30 min) and the lysosomal inhibitor (Chloroquine, Chl, 10 μM,30 min) increase NGB protein levels. Note that E2 rapidly (4 h) increases NGB protein levels, but the hormone does not increase the NGB amount in MCF-7 pre-treated with the degradation inhibitors (Fig. 3.3 A). On the contrary, the MCF-7 cell pre-treatment with the translational inhibitor (Cycloheximide, Cxe, 10 μM, 30 min) completely prevents the E2 effect on NGB levels (Fig. 3.3 B). These results, confirmed in T47D cells (Fig. 3.3 C), indicate that the E2/ERα activated rapid accumulation of NGB levels requires protein translation and the block of its degradation.

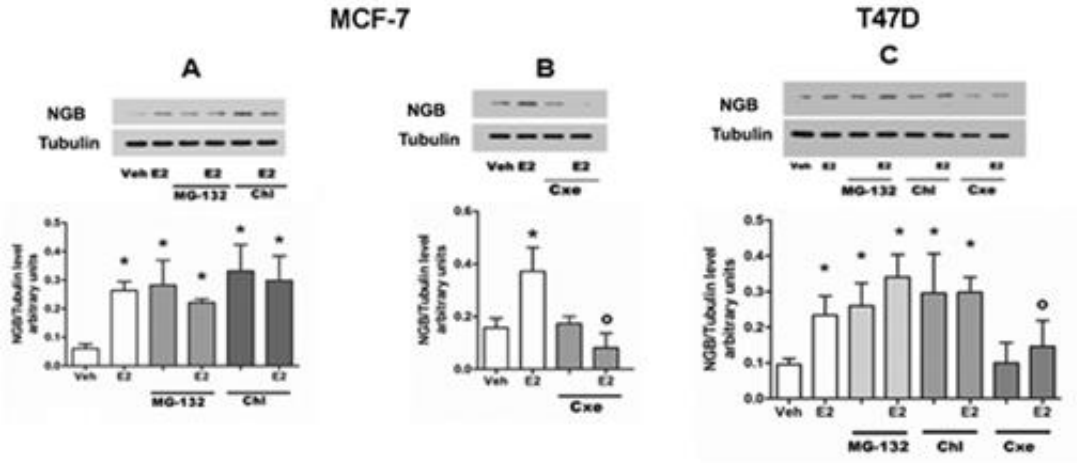

Figure 3.3 Proteasomal and lysosomal modulation of E2-induced NGB level. (A, B) MCF-7 cells and **(C)** T47D cells were pre-treated for 30 min with the proteasomal inhibitor, MG-132 (1 μM), the lysosomal inhibitor, Chloroquine (Chl, 10 μM), and the translational inhibitor Cycloheximide (Cxe, 10 μM) before E2 (10 nM) treatment for 4 h. The amount of NGB was normalized to tubulin levels. Data are means ± SD of three different experiments. $P<0.05$ was determined with Student t-test vs. vehicle (*) or vs. E2 treated samples (°).

3.2.4 Signaling pathways involved in E2-induced NGB up-regulation

The results reported in Fig. 3.3 prompted us to dissect the signal pathways involved in E2-induced NGB accumulation by evaluating the intracellular kinases involved. A library of 87 inhibitors of at least 30 individual protein kinases has been used. The screening was performed in MCF-7 cells that, among other ERα positive breast cancer cells, showed the maximum correlation between ERα and NGB up-regulation. Compounds that inhibited by at

least 80% the E2-dependent increase of NGB levels at 1 and/or 10 µM, based on the reported average IC_{50}, were defined as positive hits (Table 3.3, black dots).

Target	Drugs	≥80% inhibition positive Screening
PI3K	NVP-BEZ235	○
	GSK 1059615	●
	AS-041164	○
	CAY10626	○
	LY294002	●
	AS-605240	●
	TGX-221	○
	AS-605240	○
	AS-252424	○
	CAY10505	○
	PI-103	○
	PIK-75	●
	AS-604850	○
	PI3-Kinase α Inhibitor 2	●
	Wortmannin	●
PDK	Leelamne	●
	Arachidonic Acid Leelamide	●
	Lauric Acid Leelamide	○
AKT	CAY 10567	○
	ML-9	●
	Triciribine	●
GSK3	BIO	●
	Indirubin-3'-monoxime	●
	SB 216763	○
	SB 415286	○
	TWS119	○
mTORC	Torin 1	○
	SMI-4a	●
	INK128	○
	PP242	○
Aurora	Phthalazinone pyrazole	●
	ZM 447439	●
PKC	1-NA-PP1	○
	Bisindolylmal cimide II	●
	Chelerythrine	●
	Bisindolylmaleimide XI	●
	Bisindolylmal cimide I	○
	Bisindolylmal cimide IV	○
	Bisindolylmal cimide V	○
	NSC 663284	○
	Gö 6983	○
	II-9	●
	Bisindolylmal cimide VIII	○
	Bisindolylmal cimide IX	○
	LY 333531	○
	D-erythro - Sphingosine C-18	●
	5- Iodotubercidin	●

Target	Drugs	≥80% inhibition positive Screening
Ca2+/calmodulin-dependent kinase	KN-62	○
	KN-93	●
	KN-92	●
p38/MAPK	Doramapimod	○
	SB 203580	○
	VX-702	●
	SB 203580	●
	PD 169316	●
	SB 202190	●
	CAY 10571	●
Casein Kinase	(S)-Glycil-H-1152	○
	Apigenin	○
	NSC 210902	○
	CAY10577	○
	CAY10578	○
SPHK	CAY10621	○
	N,N-Dimethylsphingosine	○
CDK	NU 6102	○
	(R)-Roscovitine	○
	CAY10554	○
	CAY10572	●
	Olomoucine	○
	DRB	○
ROCK	CAY10622	○
	Y-27632	○
	(S)-II-1152	●
	HA-1077	●
ERK	CAY10561	●
IKK	SC-514	○
	CAY10575	●
MAPKK1	PD 98059	○
Src	PP2	●
	SU 6656	○
Raf	BAY-43-9006	○
	ZM 336372	○
JAK	AG-490	○
	Janex 1	○
JNK	SP 600125	○
	CCT018159	○
Hsp90/TBARS	Myricetin	○
EGFR/HER2	Gefitinib	○

Table 3.3 List of kinase inhibitors used in MCF-7 cells. Black and white dots indicate, respectively, compounds able or not able to inhibit more than 80% the E2 effects on NGB up-regulation. Blackdots = Positive hits; White dots = negative hits.

Successively, the identified hit compounds were grouped into clusters based on their reported primary kinase target. The E2-dependent NGB up-regulation was impaired by at least one inhibitor of phosphatidylinositol 3-kinase (PI3K), phosphoinositide dependent kinase (PDK), AKT, protein kinase C (PKC), Ca^{2+}-calmodulin dependent protein kinase II (CAMKII), p38/mitogen activated protein kinase (MAPK), and extracellular signal-regulated kinase (ERK) (Table 3 black dots). Conversely, the primary screening led to exclude the involvement of diverse kinases including Casein Kinase, Sphingosine kinase, JAK, and JNK in the E2-dependent NGB up-regulation (Table 3.3 white dots). As a whole, just 34 compounds (i.e., 39% of the total library, Fig. 3.4 red portion of the pie diagram) prevented the E2-induced NGB up-regulation.

More than half of these compounds (i.e., 56% of active inhibitors, Fig. 3.4) impaired the activity of kinases upstream and/or downstream AKT pathway. These data enlarge and confirm the preliminary data previously obtained (Fiocchetti et al., 2014).

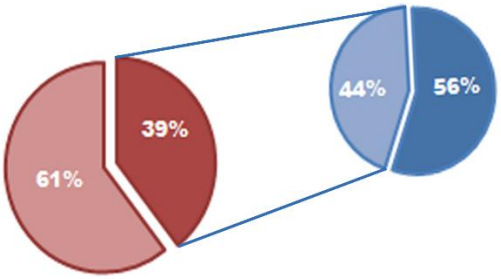

Figure 3.4 Pie diagrams depicting the percentage of negative (red portion of the right pie diagram) and positive (light blue portion of right pie diagram) hits of kinases inhibitors library and the percentage of positive hits which affect PI3K/AKT pathway (orange portion of the right pie diagram).

Although ERK and p38/MAPK pathways are pivotal components of the intricate E2-activated intracellular signals in cancer (Acconcia and Marino, 2011), here the involvement of the different components of the PI3K/AKT signaling on modulation of NGB levels has been validate being AKT activation crucial for cells survival (Fiocchetti et al., 2015; Fiocchetti et al., 2014). In particular, MCF-7 cells were treated with Wortmanin, PI3K inhibitor (Wort; 1 µM), or Arachidonic Acid Leelamide, PDK inhibitor (AAL; 1 µM), 30 min prior to the stimulation with E2 (10 nM; 24 h). As reported in Fig. 3.5 A, both of these inhibitors completely impair the E2-dependent NGB modulation confirming the involvement of these two upstream molecules in the regulation of protein levels. Furthermore, the blockade of PKC isoforms, which upstream regulate AKT activation, with Bisindolymaleimide XI (BIM XI, 1 µM, 30 min before E2) significantly impairs the E2 (10 nM, 24 h) effect on NGB level (Fig. 3.5 B). In addition, E2 completely lose its ability to increase NGB level in MCF-7 cells pre-

treated with the most active AKT inhibitor Triciribine (Tric, 1 µM) which displays the highest degree of specificity for all AKT isoforms (i.e., 1, 2, and 3) (Fig. 3.5 B) confirming the data obtained with less specific inhibitors.

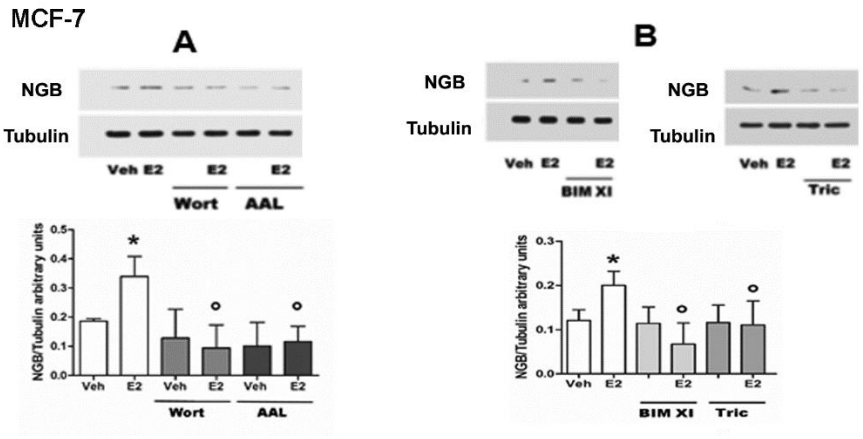

Figure 3.5 Impact of intracellular signaling proteins on E2-induced NGB up-regulation in MCF-7. Western blot analysis of NGB protein levels in MCF-7 cells treated with the **(A)** PI3K inhibitor Wortmanin (Wort, 1 µM, 30 min pre-treatment) or PDK inhibitor Arachidonic Acid Leelamide (AAL, 1 µM, 30 min pre-treatment), or **(B)** with the PKC inhibitor Bisindolymaleimide XI (BIM XI, 10 µM, 30 min pretreatment) or AKT inhibitor Triciribine (Tric, 1 µM, 30 min pretreatment) in presence or absence of E2 (10 nM) stimulation. The amount of protein was normalized by comparison with tubulin levels. Top panels are representative western blots of three independent experiments, bottom panels are results of densitometric analysis. Data are means ± S.D. of three different experiments. P<0.001 was determined with ANOVA test versus vehicle (*) and versus the E2-treated sample (°).

These results, confirmed in T47D cell line (Figs. 3.6 A, B, C), sustain the involvement of AKT pathway in the pathophysiology of ERα positive breast cancers.

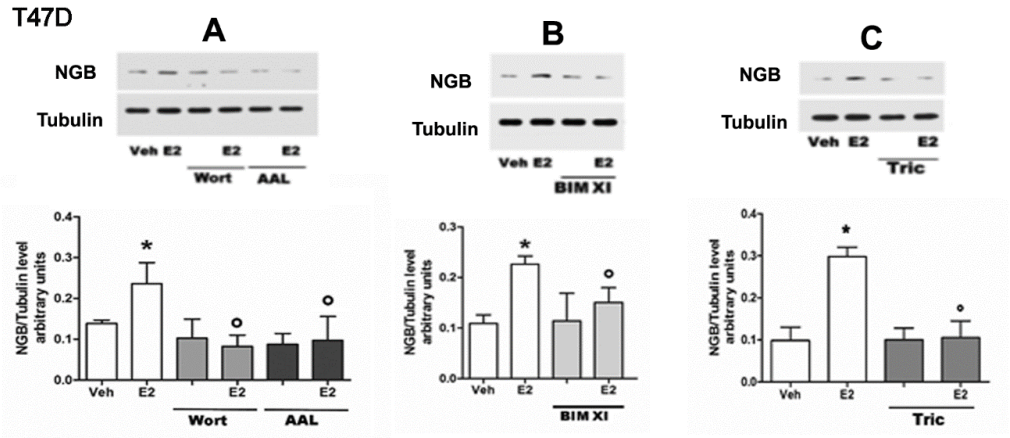

Figure 3.6 Impact of intracellular signaling protein on E2-induced NGB up-regulation in T47D. Western blot analysis of NGB protein levels in T47D cells treated with the **(A)** PI3K inhibitor Wortmanin (Wort, 1 µM, 30 min pre-treatment) or PDK inhibitor Arachidonic Acid Leelamide (AAL, 1 µM, 30 min pre-treatment), or **(B)** with the PKC inhibitor Bisindolymaleimide XI (BIM XI, 10 µM, 30 min pre-treatment) or **(C)** AKT inhibitor Triciribine (Tric, 1 µM, 30 min pre-treatment in presence or absence of E2 (10 nM) stimulation. The amount of protein was normalized by comparison with tubulin levels. Top panels are representative western blots of three independent experiments, bottom panels are results of densitometric analysis. Data are means ± S.D. of three different experiments. P<0.001 was determined with ANOVA test versus vehicle (*) and versus the E2-treated sample (°).

Intriguingly, high level of AKT phosphorylation are reported in ERα negative SK-BR-3 (Fig. 3.7) and could explain the high basal amount of NGB found in this cell line.

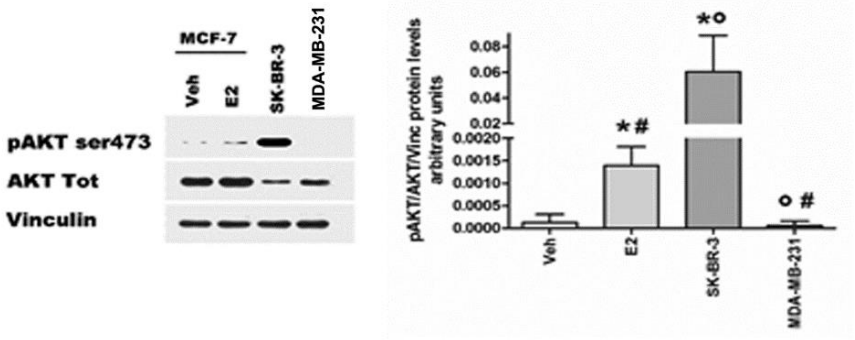

Figure 3.7 Activation of AKT in MCF-7 and ERs negative cell lines. Western blot analysis of Ser473 phosphorylation of AKT in either vehicle or E2 (10 nM, 24 h) treated MCF-7 and in vehicle treated SK-BR-3 and MDA-MB-231. The amount of protein was normalized by comparison with total AKT levels and then with vinculin levels. Right panel is a representative western blot of three independent experiments, left panel show the result of densitometric analysis. Data are means ± S.D. of three different experiments. P<0.001 was determined with ANOVA test versus MCF-7 vehicle (*), versus SK-BR-3 (°) and versus MCF-7 E2-treated sample (#).

It is well known that several transcription factors are the down-stream targets of the PI3K/AKT pathway including cyclic AMP response element-binding protein (CREB) (Du and Montminy, 1998; Song et al., 2005). Notewhorty, E2 exerts stimulatory effects on CREB protein (Pesiri et al., 2015; Yune et al., 2008) and *NGB* promoter region contains the binding sites for CREB (Cutrupi et al., 2014; Li et al., 2011; Liu et al., 2012). Thus, the involvement of CREB in E2-induced NGB up-regulation has been assessed. As previously reported in MCF-7 cells, E2 (10 nM) treatment increases CREB phosphorylation at Ser133 after 30 min and this effect was further enhanced 1 h after the hormone stimulation (Fig. 3.8 A). Furthermore, as reported in Fig. 3.8 B, 30 min of MCF-7 cells pre-treatment with the CREB inhibitor KG-501 (5 µM) before E2 stimulation (10 nM, 24 h) impaired the hormone-dependent up-regulation of NGB. Notably, NGB protein levels were significantly reduced with respect to the control cells by KG-501 treatment either in the presence or in the absence of E2 stimulation, sustaining a key role of CREB in the regulation of basal expression of NGB in these cells. Consistent with these results, the E2-dependent NGB up-regulation was dampened by KG-501 even in T47D cells, although the basal protein level was not changed possibly because different other factors could potentially modulate the NGB expression in T47D resting cells (Fig. 3.8 C).

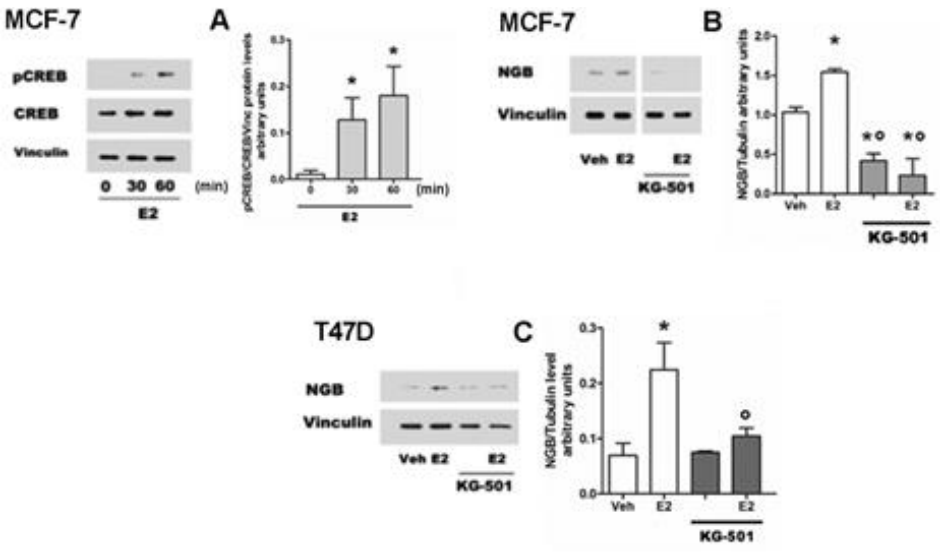

Figure 3.8 Involvement of CREB in NGB up-regulation in MCF-7 and T47D cells. (A) The phosphorylation of the Ser113 residue of CREB (pCREB) was determined by western blot in MCF-7 cells treated with E2 (10 nM) for 30 or 60 min. The nitrocellulose was stripped and then probed with anti-CREB and anti-vinculin antibodies. **(B)** Analysis of NGB protein levels in MCF-7 cells pretreated for 30 min with CREB inhibitor KG-501 (5 μM) and then stimulated with either vehicle or E2 (10 nM). **(C)** Analysis of NGB protein levels in T47D cells pretreated for 30 min with CREB inhibitor KG-501 (5 μM) in presence or absence of E2 stimulation. The amount of protein was normalized by comparison with vinculin levels. Data are means ± S.D. of three different experiments. P<0.001 was determined with ANOVA test versus vehicle (*) and versus the E2-treated sample (°).

ERα dependent activation of CREB takes part to the E2 activated pathway, which lead the up-regulation of the anti-apoptotic protein Bcl-2 (Pesiri et al., 2015; Yune et al., 2008). Recently, we demonstrated that, in NGB silenced MCF-7 cells, E2 loses its ability to modulate Bcl-2 protein levels (Fiocchetti et al., 2014). Consequently, the effect of previous defined kinases inhibitors on the E2-induced Bcl-2 protein has been evaluated. As shown in Fig. 3.9 A, the positive modulation of E2 (10 nM, 24 h) on Bcl-2 protein level is significantly reduced in MCF-7 cells pre-treated with Wortmanin (1 μM; 30 min) and completely blocked in the presence of the PDK inhibitor AAL.

Similarly, E2 does not increase Bcl-2 protein expression after PKC and AKT protein cascade and CREB blockade (Figs. 3.9 B and C) sustaining a close relationship between the E2-dependent up-regulation of NGB and the hormone effect on the regulation of the Bcl-2 protein levels.

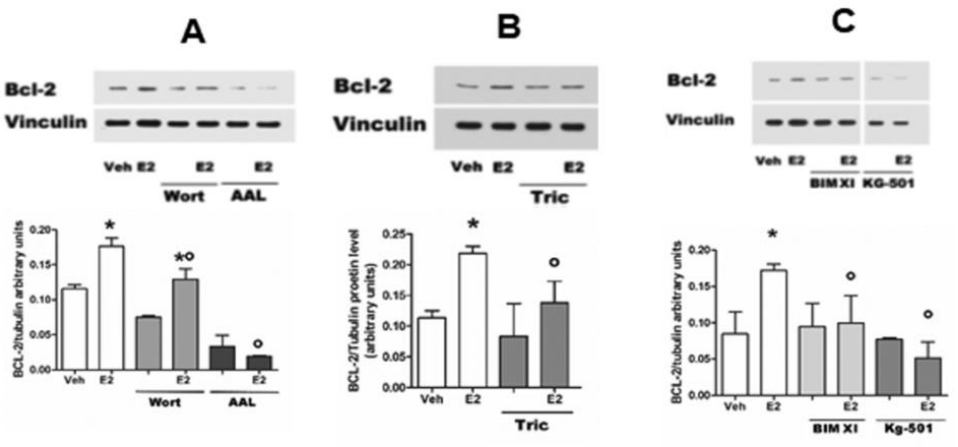

Figure 3.9 Impact of intracellular signaling protein on Bcl-2 in MCF-7. Western blot analysis of Bcl-2 protein levels were performed in MCF-7 stimulated with either vehicle or **(A)** PI3K inhibitor Wortmanin (Wort, 1 µM, 30 min), PDK inhibitor Arachidonic Acid Leelamide (AAL, 1 µM, 30 min), **(B)** AKT inhibitor Triciribine (Tric, 1 µM, 30 min), **(C)** PKC inhibitor Bisindolymaleimide XI (BIM XI, 10 µM, 30 min) or CREB inhibitor KG-501 (5 µM, 30 min) followed by 24 h stimulation with E2 (10 nM). The amount of protein was normalized by comparison with vinculin levels. Top panels are representative western blots of five independent experiments. Bottom panels show results of denstitometric analysis. Data are means ± S.D. of five different experiments. P<0.001 was determined with ANOVA test versus vehicle (*) and versus the E2-treated sample (°).

3.2.5 Involvement of the AKT pathway in E2-induced NGB mitochondrial localization

The NGB localization into the mitochondria appears to be crucial for the anti-apoptotic function of the globin in cancer cell lines (Fiocchetti et al., 2015; Fiocchetti et al., 2014). Here, we assess the ability of E2 to reallocate NGB in mitochondria in ERα containing MCF-7 and T47D cells by analyzing the NGB co-localization with the mitochondrial marker COX-IV after 4 h of E2 treatment (10 nM) (Fig. 3.10 A). Confocal image analyses with the 8.2 IMARIS software reveals that E2 significantly increase the NGB-COX-IV merged signals in both MCF-7 and T47D cells (Fig. 3.10 B), confirming a common mechanism by which the hormone affect NGB mitochondrial localization in ERα-containing breast cancer cells. Intriguingly, the data obtained by mitochondrial fractionation in MCF-7, SK-BR-3 and MDA-MB-231 (Fig. 3.10 C) show that the ERs negative SK-BR-3 cell line exhibits a major amount of NGB into mitochondria, as compared to MDA-MB-231 (ERs negative) and MCF-7 (ERα positive) cell lines, in line with the high activation of AKT found in these cells (see Fig. 3.7).

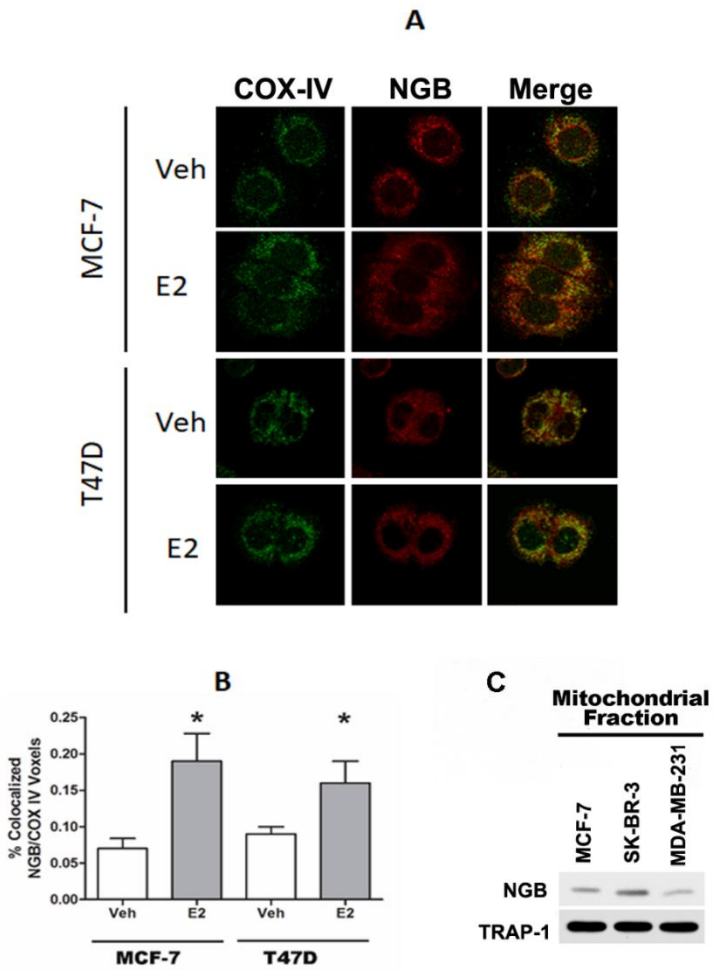

Figure 3.10 NGB mitochondrial localization. (A) Confocal microscopy analysis of NGB and COX-4 co-immuno-localization. Cells were fixed, permeabilized, and stained with anti-NGB antibody (red) and co-stained with anti-COX4 antibody (green) (original magnification × 63). Merged images by confocal microscopy show the mitochondrial localization of NGB NGB distribution in MCF-7 and T47D cells treated with either vehicle or E2 (10 nM) for 4 h. All images are single Z-stack planes and are representative of three independent experiments. **(B)** Results of quantitative analysis of percentage of NGB and COX-4 colocalization voxels performed with Imaris 6.3.1 software. Data are means ± S.D. of three different experiments. P<0.05 was determined with ANOVA test versus vehicle (*). **(C)** Western blot analysis of NGB and TRAP-1 (mitochondrial marker) in mitochondrial fraction of MCF-7, SK-BR-3 and MDA-MB-231 cells treated with vehicle for 24 h.

Moreover, the NGB and COX-IV co-localization in MCF-7 cells treated with the previously validated kinases inhibitors 30 min prior to E2 (10 nM, 4 h) stimulation (Fig. 3.11 A) has been evaluated. Intriguingly, the PI3K, PDK, and AKT signaling blockade by Wortmanin (Wort, 1 µM), Arachidonic Acid Leelamide (AAL, 1 µM), and Triciribine (Tric, 1 µM), respectively, completely prevented E2-induced increase in NGB-COX-4 co-localization (Figs. 3.11 A and B). Conversely, E2 was still able to increase NGB-COX-4 merged signals in MCF-7 cells pre-treated with the PKC inhibitor BIM XI (Figs. 3.11 A and B). Taken together, these results suggest that the activation of the PI3K/PDK/AKT axis is needed for the E2-dependent re-localization of NGB to mitochondria.

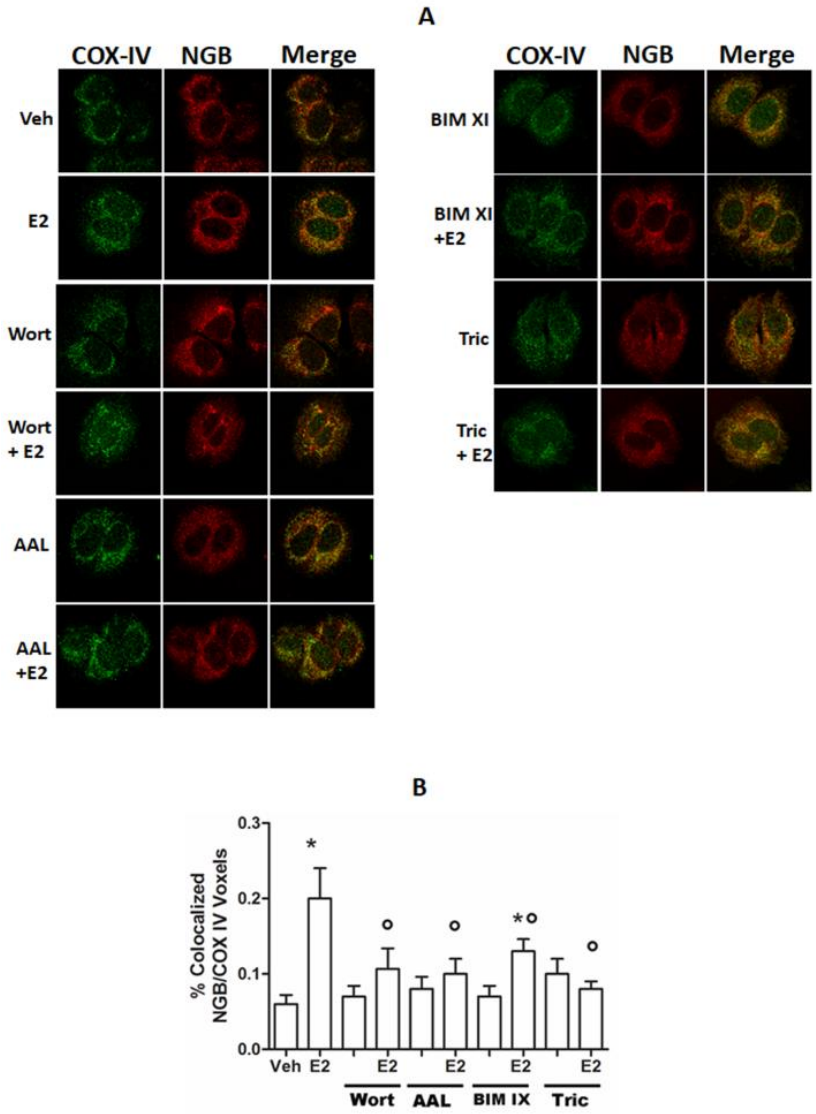

Figure 3.11 Impact of intracellular signaling protein on E2-induced NGB mitochondrial localization. (A) Confocal microscopy analysis of NGB and COX(IV) co-localization in MCF-7 cells pretreated with Wort (1 μM), AAL (1 μM), Tric (1 μM) or BIM XI (10 μM) 30 min before the E2 (10 nM) stimulation for 4 h. Cells were fixed, permeabilized, and stained with anti-NGB antibody (red) and co-stained with anti-COX4 antibody (green) (original magnification × 63). All images are single Z-stack planes and are representative of three independent experiments. **(B)** Quantitative analysis results relative to the percentage of NGB and COX(IV) colocalized voxels by using Imaris 6,3,1 software. Data are means ± S.D. of three different experiments. $P<0.05$ was determined with ANOVA test versus vehicle (*).

3.3 Discussion

Breast cancer shows a high heterogeneity and originally the classification of breast cancers was founded on their histopathological type and grade, but also the expression of three cancer biomarkers, ERα, Her2/Neu and PR, has been considered (Vuong et al., 2014). We analysed ERα positive ductal carcinoma specimens, in particular selected from 55-75 years old patients, in postmenopausal age that is characterized by circulating low estrogen levels. First, we studied the expression of cancer biomarkers. The data reported show the

amplification of the Her2/Neu receptor and ERα in all breast tumor samples, while high expression of PRA and PRB are shown in only one sample. Moreover, all breast tumor samples show the expression of pERα; this data is in line with literature that assessed that at the postmenopausal age the adipose tissue becomes the principal site of estrogen biosynthesis (Savolainen-Peltonen et al., 2014). Conversely, EGF receptor is highly expressed in normal tissues, in line with literature data (Dittadi et al., 1993). As discussed previously, the correlation between NGB levels and cancer is still unclear (Emara et al., 2010; Gorr et al., 2011). The data reported show the expression of both NGB in tumoral samples but not in normal ones, confirming a key role of the globin in breast cancer physiology. Moreover, Pearsons's coefficient show a strong correlation between NGB and ERα. Furthermore, we analyzed Bcl-2 expression. All breast tumor samples show high Bcl-2 level and Pearsons's coefficient show a strong correlation between NGB and Bcl-2. In order to obtain a more precise correlation between NGB level and ERα activation, we considered five breast cancer cell lines, characterized by a different ERα expression. Although NGB levels are present at the basal level in all the cell lines, E2 dependent up-regulation of NGB is maintained only in ERα-containing breast cancer cell lines (i.e., MCF-7, T47D, ZR-75-1). On the other hand, no E2 effects were reported in ERs negative cells (i.e., SK-BR-3 and MDA-MB-231) confirming a closed relationship between the expression of ERα and modulation of NGB. Moreover, the involvement in the E2/NGB pathway of GPER, a novel estrogen receptor which seems to contribute to the signaling of E2 to the plasma membrane (Maggiolini and Picard, 2010; Prossnitz and Barton, 2011) and of EGFR, which cross-talks with the ERα rapid signals (Kahlert et al., 2000; Pietras, 2003; Song et al., 2007), is not evidenced. Finally, a role of ERβ subtype in modulation of NGB in breast cancer cells should be ruled out due to the low expression level of this receptor subtype in breast cancer. Few *in vivo* experiments indicate that the ERα expression increases in early breast cancers from the low-grade to high-grade ductal carcinoma in situ and then declines in more invasive cancers (Thomas and Gustafsson, 2011). Conversely, a gradual reduction of ERβ has been observed from normal to pre-invasive lesions and invasive carcinomas probably due to the methylation of ERβ promoter (Thomas and Gustafsson, 2011). Taken together, these data strongly sustained our hypothesis confirming a closed relationship between the activation of ERα and the E2-induced NGB over-expression in breast cancer. Intriguingly, an E2-independent NGB up-regulation may occur. Indeed, the ERα negative SK-BR-3 contain high amount of NGB, which is not under

E2 regulation suggesting that an active signal pathway, probably shared with those triggered by E2 but not mediated by the above described receptors, should exist in this cell line.

The discrepancies in NGB levels and intracellular localization obtained in SK-BR-3 cells as well as in E2-stimulated cells, prompted us to verify if different signaling pathways are at the root of these divergences. E2 exerts, *via* ERα, both rapid extra-nuclear and long-term genomic functions (Fiocchetti et al., 2014). In particular, the evidence that NGB mRNA and protein increases 4 and 2 h, respectively, after the E2 treatment together with the absence of any ERE box in the *NGB* promoter (Cutrupi et al., 2014; Fiocchetti et al., 2014) indicates that NGB up-regulation should depends on rapid mechanism, which control the protein levels. Here, we demonstrate that E2 increases NGB level by preventing the protein degradation as demonstrated by the inhibition of lysosomal and proteasomal pathways, which accumulate NGB into the cells. The E2 inability to further increase NGB level after degradation pathway inhibition strongly sustain that hormone induces NGB up-regulation by preventing its degradation. In addition, the experiments with cycloheximide, an inhibitor of protein translation, support that the activation of protein translation is necessary for E2-dependent NGB up-regulation. In summary, both the impairment of protein degradation and the enhancement of protein synthesis are at the root of E2-dependent rapid NGB up-regulation.

In order to define the signaling pathways, which affect NGB protein levels modulation and localization, a library of intracellular protein kinases inhibitors has been tested in MCF-7 cells. The screening analysis evidences that 34 kinases were involved in the E2 modulation of NGB expression including PI3K/AKT, PKC, CAMKII, Aurora kinase, p38/MAPK and ERK. Although several of these pathways have been closely associated to the E2/ERα effects in breast cancer cells (Acconcia and Marino, 2011; Marino et al., 2006), the E2-induced PI3K/AKT pathway activation transduces the E2 anti-apoptotic and pro-survival effects in diverse cancer cell lines (Acconcia and Marino, 2011; Marino et al., 2006). In particular, the PI3K/AKT pathway maintains the inactivation of the pro-apoptotic BAD protein (Acconcia and Kumar, 2006; Fernando and Wimalasena, 2004) and avoids the cleavage of caspase-3 increasing the expression of the anti-apoptotic protein Bcl-2 (Acconcia et al., 2005b; Bratton et al., 2010). These results led us to validate the involvement of PI3K/AKT pathway in the modulation of NGB protein level and localization. Both PI3K/PDK/AKT pathway and PKC activation, an upstream activator of AKT (Urtreger et al., 2012), are required for the E2-induced increase of NGB levels in both MCF-7 and T47D cells, as well as for the

overexpression of Bcl-2 protein, which increase requires high NGB level (Fiocchetti et al., 2014).

As a key cellular regulator that transduces various signals, the activity of AKT is dynamically regulated. Upon hormone and growth factor stimulation, active PI3K recruits AKT to plasma membrane where it is phosphorylated on two key residues: Thr308 by PDK1 and Ser473 by mammalian target of rapamycin–Rictor complex leading to full activation of AKT. Therefore, E2 stimulation of breast cancer cells triggers the PI3K/PDK-dependent AKT Ser473 phosphorylation. In particular, E2 is able to rapidly and persistently activate AKT phosphorylation (Acconcia et al., 2005b) and the pathway culminates in NGB up-regulation and re-allocation into mitochondria. The high NGB level in whole cells and mitochondria paralleled with the high AKT phosphorylation status found in ERα negative and Her2/Neu positive SK-BR-3 cells strongly sustain the role of AKT. To further confirm the pivotal role of AKT in NGB accumulation, no activation of AKT and barely detectable level of NGB are detected in the triple negative MDA-MB-231 cell line. Finally, the AKT activation converges on the transcriptional regulation of anti-apoptotic genes (Hennessy et al., 2005). Our result indicate the crucial role of transcription factor CREB (one of the AKT downstream transcriptional factors) in the E2-induced modulation of NGB levels in both MCF-7 and T47D cells. Therefore, the rapid and persistent E2/ERα-induced AKT assure the rapid NGB accumulation into the cells, the long-term transcription of *NGB* gene, and the translocation of NGB into mitochondria where NGB exerts its role in promoting cell survival and avoiding the triggers of apoptotic cascade (Fiocchetti et al., 2015; Fiocchetti et al., 2014).

Findings reported here introduce a new insight in the pathophysiology of breast cancer defining NGB as a conserved compensatory protein induced by E2 and identifying the ERα activated PI3K/AKT signaling as the main intracellular upstream pathway by which E2 affects NGB compartmentalization into mitochondria (Fig. 3.12).

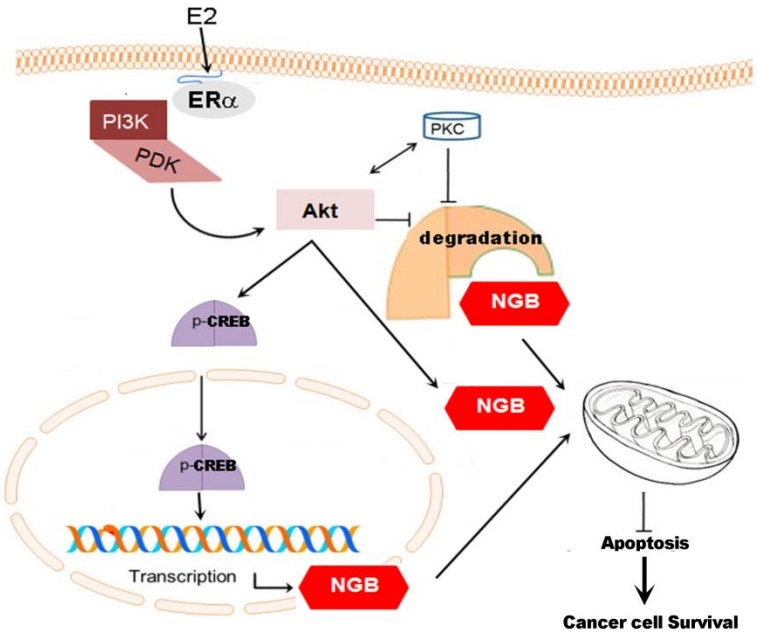

Figure 3.12 Graphic representation of ERα/PI3K/AKT pathway by which E2 affects NGB compartmentalization into mitochondria.

4. ROLE OF NGB IN BREAST CANCER

4.1 Introduction

NGB over-expression, driven by transiently transfected pcDNA vector, protects cultured neurons against hypoxia (Sun et al., 2001), enhances neuron survival under anoxia or oxygen/glucose deprivation (Fordel et al., 2007), and displays neuro-protective properties against hypoxic/ischemic and oxidative stress (Fiocchetti et al., 2013; Greenberg et al., 2008; Yu et al., 2009; Yu et al., 2012a). In addition, in brain derived cell lines, hypoxia (Brunori et al., 2005), H_2O_2 injury (De Marinis et al., 2013b), and lipopolysaccharides (De Marinis et al., 2013a) moderately induce NGB. This suggests that NGB could behave in neurons as a sensor of injuring stimuli including oxidative stress, hypoxia, and neurotoxicity. However, as described in detail previously, no differences in NGB levels in murine models of traumatic brain injury, experimental autoimmune encephalitis, cerebral malaria, and hypoxia have been found (Burmester and Hankeln, 2009; DellaValle et al., 2010; Hundahl et al., 2006; Li et al., 2006), thus insinuating uncertainty on the role of NGB as a stress sensor.

The experiments reported in chapter 3 clearly indicated that ERα positive ductal carcinoma tissues are characterized by higher NGB levels with respect to the non-cancerous counterpart. As oxidative stress and hypoxia are conditions frequently occurring in fast proliferating neoplastic tissues, it is possible that NGB may be part of the defence mechanism established by cancer cells to counteract tumor environment stress condition by helping cells to survive (Emara et al., 2010; Oleksiewicz et al., 2011). Indeed, cancer cells adapt themselves to the stressful and dynamic microenvironment of solid tumors, where the redox status is imbalanced and oxygen/nutrients availability is limited (Cairns et al., 2011; Gorrini et al., 2013). The adaptation is achieved by developing alternative compensatory metabolic reactions that render cancer cells insensitive to stress inducers such as chemotherapy and radiation (Gorrini et al., 2013). This evidence rises the possibility that high level of NGB could act in breast cancer cells, like in neurons, as a compensatory protective protein activated in response to injuring stimuli and able to prevent mitochondria-dependent apoptosis. To evaluate this hypothesis the effect of different stressors on the level, localization, and function of NGB in wild-type or NGB stable silenced breast cancer cells, has been tested. In particular the effect of hypoxia, nutrient deprivation, hydrogen peroxide (H_2O_2), lead (IV) acetate

(Pb(IV)) and paclitaxel was evaluated. E2 treatment has been used as a positive control of NGB up-regulation and MCF-7 cell survival.

4.2 Results

4.2.1 Effect of hypoxia on NGB levels

The effect of hypoxia has been evaluated by exposing MCF-7 cells in special hypoxic chambers (kindly provided by Prof. Fabio Carraro, University Siena) in which the partial O_2 pressure was setted at ~14 mmHg (2%).

Neither 24 h (Fig. 4.1) nor 48 h (data not shown) of hypoxia (2% O_2) increases NGB protein levels in comparison to normoxia (21% O_2) in MCF-7 cells. However, E2 treatment (10 nM, 24 h) still induces the up-regulation of NGB and the accumulation of the hypoxia biomarker HIF1α (Fig. 4.1).

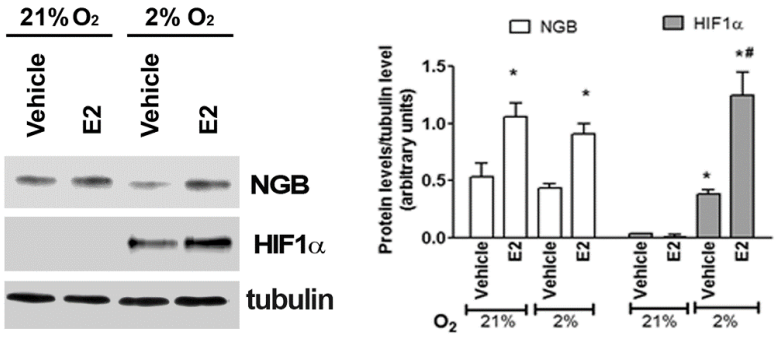

Figure 4.1 Effect of hypoxia in MCF-7 cells. NGB and HIF1α protein expression in MCF-7 cells exposed to either normoxia (21% O_2; 24 h) or hypoxia (2% O_2; 24 h), in the presence and absence of E2 (10 nM; 2 h pretreatment). The amount of proteins were normalized to tubulin levels. Right panel is typical Western blot of three independent experiments. Left panel represents the results of the densitometric analysis. Data are means ± SD of three different experiments. $P<0.05$ was determined with Student t-test vs. normoxia vehicle (*) and vs. hypoxia vehicle (#).

4.2.2 Effect of nutrient deprivation on NGB levels

To dissect the effects of nutrient deprivation on NGB levels, MCF-7 cells were cultured in Earle's Balanced Salt Solution (EBSS), a medium characterized by a low glucose concentration and amino acids and serum free in order to mimic the nutrient deprivation environment. E2 treatment (10 nM) induces NGB up-regulation at 4 and 6 h but not at 2 h (Fig. 4.2), while EBSS up-regulates NGB protein levels at all time considered (2, 4 and 6 h) Moreover, the concomitant administration of E2 and EBSS increases NGB level in the same manner of EBSS or E2 treatment.

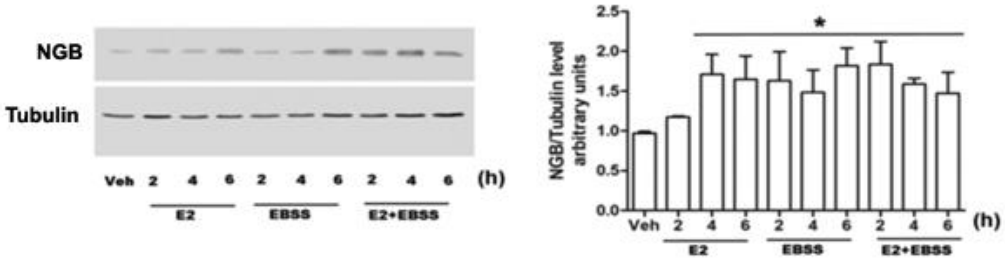

Figure 4.2 Effect of nutrient deprivation on NGB levels. Western blot (right) and densitometric analyses (left) of NGB protein levels in MCF-7 cells treated with the vehicle (ethanol/PBS 1/10), E2 (10 nM), and Earle's Balanced Salt Solution (EBSS) or E2 (10 nM, 30 min before) and EBSS at different time (2, 4, 6 h). The amount of proteins were normalized to tubulin levels. Data are the mean ± SD of five different experiments. P<0.001 was determined with Student's t-test with respect to the vehicle (*).

4.2.3 Effect of oxidative stress-inducing compounds on NGB levels

To determine the role of NGB during oxidative stress injury, MCF-7 cells have been treated with H_2O_2 (a ROS) and Pb(IV) (a pollutant which induces oxidative stress and mitochondrial-dependent apoptosis) (Kahlert et al., 2000). E2 treatment has been used as a positive control. Neither vehicle nor E2 (10 nM) enhanced ROS level in MCF7 cells, whereas cell treatment with either H_2O_2 (400 μM) or Pb(IV) (200 μM) increased the ROS production reaching maximum levels after 30 min of treatment (14.68 ± 0.04 and 11.00 ± 1.49 fold over the control, respectively) (Fig. 4.3 A).

Finally, the capability of E2, H_2O_2, and Pb(IV) to modify the NGB expression by valuating NGB mRNA (Fig. 4.3 B) and protein level (Figs. 4.3 C, D) has been evaluated. E2 induces the increase of NGB mRNA 4 h after treatment (Fig. 4.3 B), whereas neither H_2O_2 nor Pb(IV) modulate NGB mRNA levels (Fig. 4.3 B). Conversely, like E2, both H_2O_2 and Pb(IV) increase NGB protein levels (Fig. 4.3 C). To quantify the results obtained by Western blot, the intensity of the NGB bands was compared with that obtained loading 5 ng of recombinant NGB (kindly provided by Cinzia Verde, CNR, Napoli). MCF-7 cells contain a very low basal level of NGB (30 ± 3.3 ng/mg protein lysate) which significantly doubles 24 h after E2 (60 ± 3.2 ng/mg protein lysate), H_2O_2 (48 ± 2.2 ng/mg protein lysate), and Pb(IV) (46 ± 2.1 ng/mg protein lysate) treatment (Fig. 4.3 D).

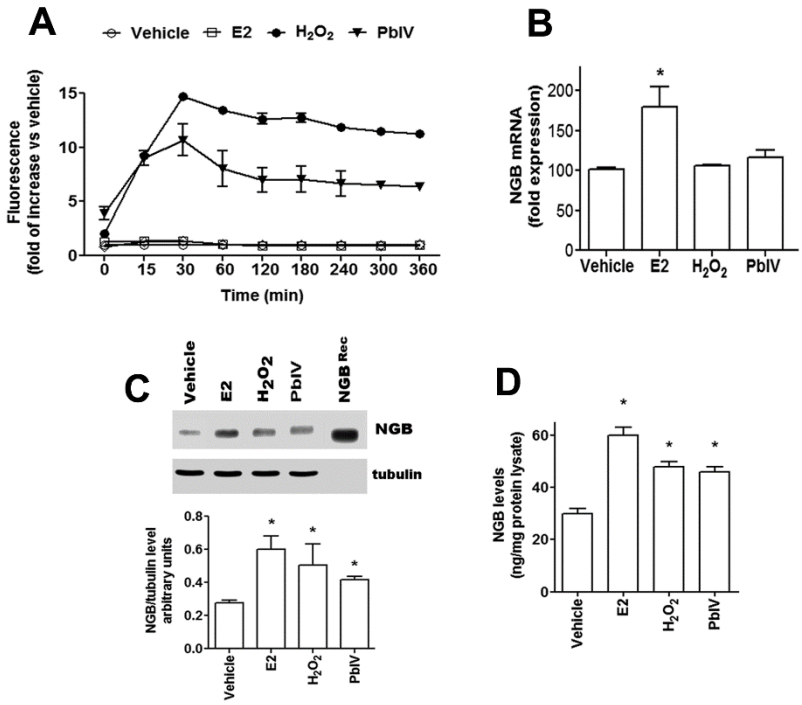

Figure 4.3 Characterization of H_2O_2 and Pb(IV) as MCF-7 cell stressors. (A) Cells were exposed to E2 (10 nM), H_2O_2 (400 μM), and Pb(IV) (200 μM). ROS measurement was obtained by DCFH-DA fluorescence analysis. **(B)** NGB mRNA levels in MCF-7 cells. The NGB expression is reported as fold of induction over the vehicle (set to 100). Data represent the mean ± SD of five different experiments. Significant differences ($p<0.001$) were determined by ANOVA followed by the Turkey-Kramer post-test with respect to unstimulated samples (*). **(C)** Analysis of NGB protein levels in cells treated with the above reported compounds for 24 h. The amount of protein was normalized to tubulin levels. Top panel is typical Western blot of three independent experiments. Bottom panel represents the result of densitometric analyses. Data are means ± SD of three different experiments. $P<0.05$ was determined with Student t-test vs. vehicle (*). **(D)** NGB protein amount in treated cells. NGB protein cell content was quantified by comparing the Western blot band intensity of treated sample NGB with the band intensity of 5 ng NGB recombinant protein used as protein standard. Data are means ± SD of three independent experiments. $P<0.05$ was determined with Student t-test vs. vehicle (*).

4.2.4 Effect of H_2O_2 and Pb(IV) on mitochondrial NGB localization

The NGB localization into the mitochondrial compartment is necessary to act as an anti-apoptotic protein in several cell lines (De Marinis et al., 2013b; Fiocchetti et al., 2015; Fiocchetti et al., 2014). This prompted us to verify if the selected compounds could modify NGB mitochondrial localization. Confocal microscopy analyses show the co-localization of NGB with the mitochondrial marker COX-4 (cytochrome *c* oxidase-4) (Fig. 4.4 A). Although the confocal microscopy allows a purely qualitative analysis, the analysis with the 8.2 IMARIS software demonstrated that only E2 treatment (24 h) significantly raises the NGB-COX-4 merged signals (Fig. 4.4 A). The increase of the NGB localization at mitochondrial level has been confirmed in isolated mitochondria by using a cell fractionation kit and Western blot. Fig. 4.4 B confirms the purity of mitochondrial fraction. In fact PP2A, cytosolic marker is absent, while TRAP-1, mitochondrial marker, is evident. As expected (Fiocchetti et al., 2014), E2 (10 nM; 24 h) increases the mitochondrial NGB content; conversely, H_2O_2

treatment (400 μM; 24 h) does not modify the NGB mitochondrial localization (Fig. 4.4 C). Intriguingly, Pb(IV) (200 μM, 24 h) treatment induces a significant decrease of the NGB amount in the mitochondrial fraction (Fig. 4.4 C).

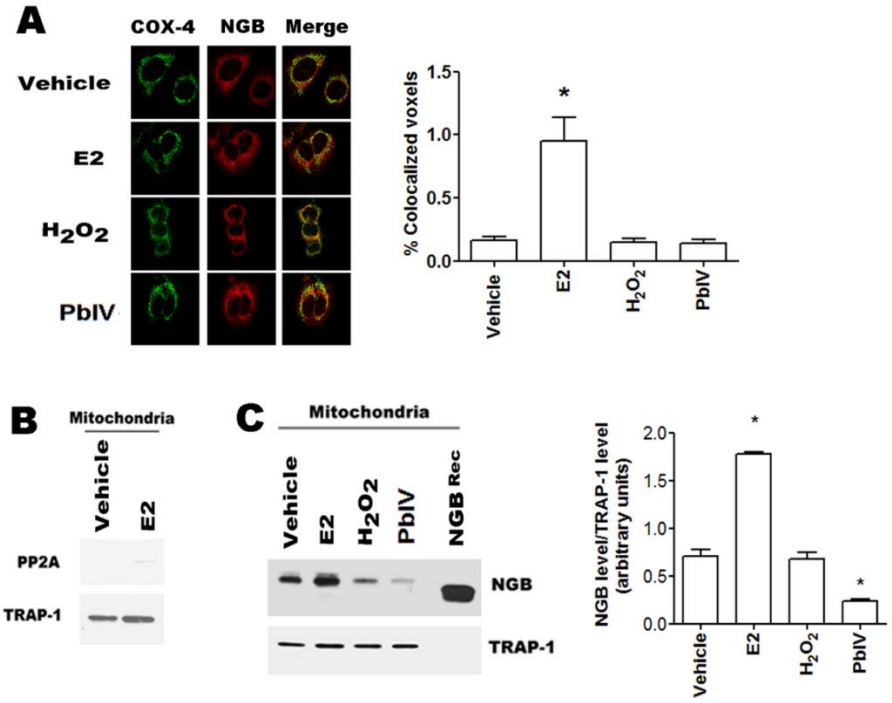

Figure 4.4 Mitochondrial NGB localization. (A) Left, Confocal microscopy analysis of NGB and cytochrome c oxidase-4 (COX-4) co-immuno-localization in MCF-7 cells treated for 24 h with vehicle and/or E2 (10 nM), H_2O_2 (400 μM) and Pb(IV) (200 μM); Right panel, quantitative analysis of co-localization. Cells were fixed, permeabilized and stained with anti-NGB antibody (red) and co-stained with anti-mitochondrial COX-4 antibody (green) (original magnification x 63). All images are single Z-stack planes and are representative of three independent experiments. **(B)** Western blot analysis of PP2A (cytosolic marker) and TRAP-1 (mitochondrial marker) in mitochondrial fraction of MCF-7 cells treated with either vehicle and/or E2 (10 nM) for 24 h. **(C)** Typical Western blot of three independent experiments of NGB expression in mitochondrial fraction of MCF7 cells treated for 24 h with the above reported compounds. Left panel is typical Western blots of three independent experiments. Right panel represents the result of densitometric analyses. The amount of proteins was normalized to the fraction marker protein TRAP-1. Data are means ± SD of three different experiments. $P<0.05$ was determined with Student t-test vs. vehicle (*).

4.2.5 Pathways involved in H_2O_2- and Pb(IV)-induced NGB up-regulation

In order to obtain clear evidence on how NGB level could be regulated by H_2O_2 and Pb(IV), MCF-7 cells were treated with either the proteasomal inhibitor MG-132 (1 μM for 30 min), the lysosomal inhibitor Chloroquine (Chl, 10 μM for 30 min), and the translational inhibitor Cycloheximide (Cxe, 10 μM for 30 min) before treatment with the ROS inducers.

Figs. 4.5 A, B and C show that NGB level is reduced by Cxe and increased by lysosomal degradation. H_2O_2 and Pb(IV) treatments do not modify this trend suggesting that ROS-inducing compounds could increase NGB levels by inhibiting lysosomal degradation and

increasing NGB translation. Intriguingly, MG-132 does not modulate NGB level, but completely impairs H_2O_2 and Pb(IV) effect in enhancing NGB level (Figs. 4.5 A, B and C). As a whole, these data indicate that ROS-inducing compounds increase NGB protein levels in MCF-7 cancer cells activating specific pathways divergent from that triggered by E2 (see chapter 3). Thus, in order to demonstrated that E2 and Pb(IV) up-regulate NGB level through two different pathways, we explored the signaling pathways reported in Chapter 3, which regulate NGB intracellular levels.

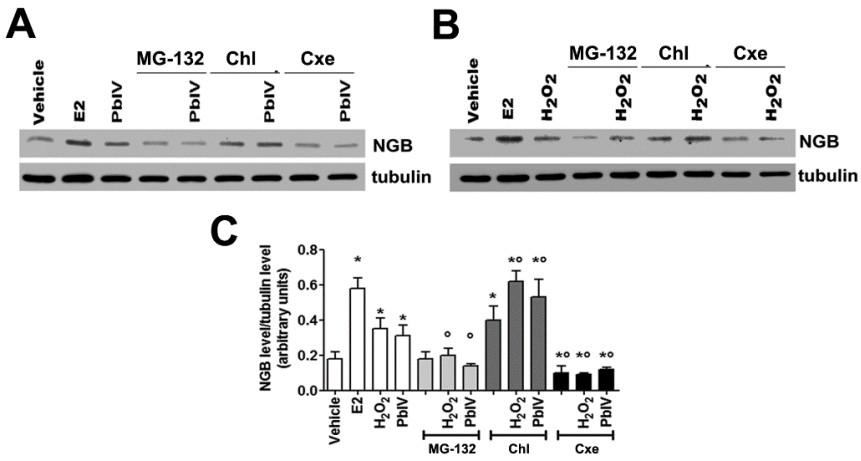

Figure 4.5 Characterization of H_2O_2 and Pb(IV) activated pathways, which affect NGB protein levels. Western blot (**A** and **B**) and densitometric analyses (**C**) of NGB protein levels in MCF-7 cells pre-treated with the proteasomal inhibitor MG-132 (10 µM), the lysosomal inhibitor Chloroquine (Chl, 1 µM) or the translation inhibitor Cyclohexemide (Cxe, 10 µM) 30 min before the stimulation **(A)** Pb(IV) (200 µM) or **(B)** H_2O_2 (400 µM) for 24 h. The amount of protein was normalized by comparison with tubulin. Data are means ± SD of three different experiments. P<0.001 was determined with Student t-test vs. vehicle (*) or vs. non-treated samples (°).

The data obtained showed that, contrarily to E2 (see Figs 3.5 A and B), PI3K and PDK kinase blockade do not interfere with the Pb(IV)-dependent increase of NGB protein levels, whereas the PKC inhibitor and AKT inhibitor Tric affect the Pb(IV)-induced NGB up-regulation (Figs. 4.6 A, B and C).

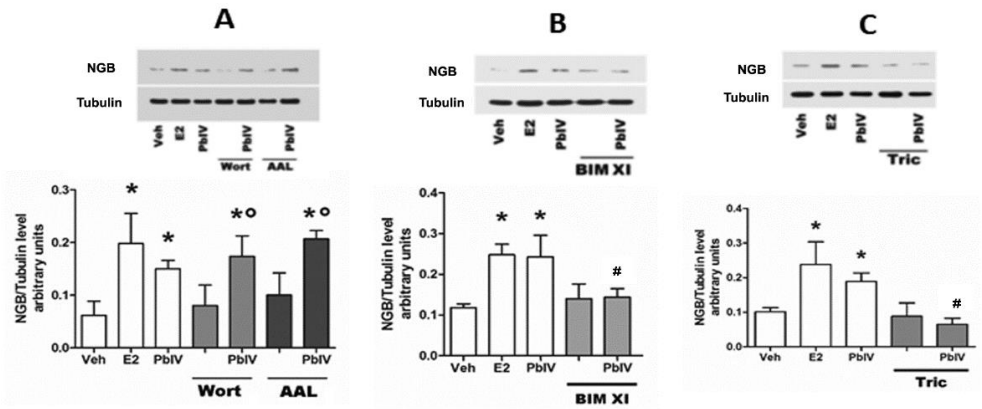

Figure 4.6 Characterization of PbIV activated pathways, which affect NGB protein levels. Analysis of NGB protein levels in MCF-7 pretreated with **(A)** PI3K inhibitor Wortmanin (Wort, 1 μM, 30 min pre-treatment) or PDK inhibitor Arachidonic Acid Leelamide (AAL, 1 μM, 30 min pre-treatment), or **(B)** with the PKCα inhibitor Bisindolymal eimide XI (BIM IX, 10 μM, 30 min pre-treatment) or **(C)** with AKT inhibitor Triciribine (Tric, 1 μM, 30 min pre-treatment) in presence or absence of PbIV (200 μM) stimulation. The NGB protein levels after E2 (10 nM) stimulation has been considered as positive control of NGB protein induction. The amount of protein was normalized by comparison with tubulin levels. Data are means ± S.D. of five different experiments. P<0.001 was determined with ANOVA test versus vehicle (*), or versus the E2-treated sample (°), or versus the Pb(IV)-treated sample (#).

This prompted us to evaluate if some differences in the timing of AKT activation might support the diverse E2 and Pb(IV) effect on the mitochondrial NGB localization. As expected, E2 (10 nM) increases AKT phosphorylation already 30 min after the hormone stimulation and this effect further increases 4 h thereafter (Figs. 4.7 A and B). Intriguingly, Pb(IV) treatment (200 μM) show a similar ability to induce the AKT phosphorylation than E2 at 60 and 120 min of stimulation (Fig. 4.7 A), while it induces a three-fold higher AKT phosphorylation at 30 min; whereas no significant AKT activation was evident after 4 h of stimulation, indicating that Pb(IV) induces the rapid and transient activation of AKT (Fig. 4.7 B).

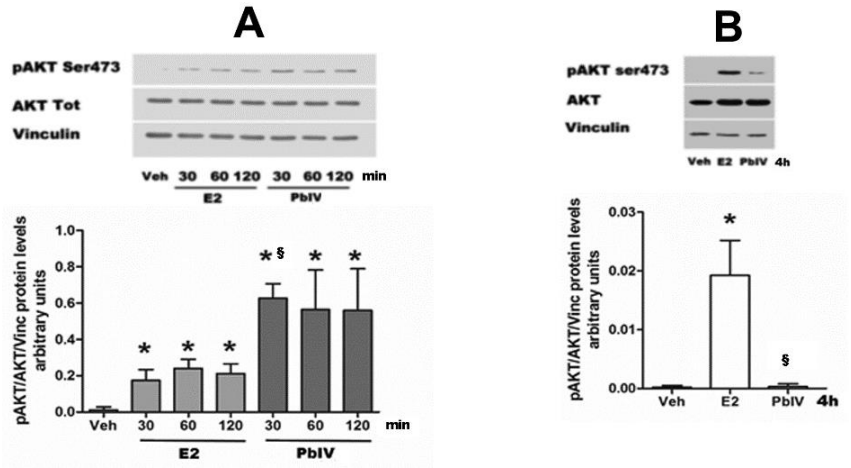

Figure 4.7 Effect of Pb(IV) on AKT phosphorylation. Ser473 phosphorylation were evaluated in MCF-7 cells treated with E2 (10 nM) or Pb(IV) (200 μM) for **(A)** 30, 60, and 120 min, or **(B)** 4 h. The amount of protein was normalized by comparison with vinculin levels. Top panels are representative western blots of five independent experiments, bottom panels show results of densitometric analysis. Data are means ± S.D. of five different experiments. P<0.001 was determined with ANOVA test versus vehicle (*) and versus the E2-treated sample (§).

As a whole these data demonstrated that divergent downstream signals and functional outcomes originates.

4.2.6 Effect of H_2O_2 and Pb(IV) on the NGB anti-apoptotic function

As previously reported (Fiocchetti et al., 2015; Fiocchetti et al., 2014), E2-induced NGB up-regulation counteracts apoptosis induced by oxidative stress in several cancer cell lines. Thus, we evaluated the anti-apoptotic role of NGB in the presence of the selected ROS-inducing compounds.

As reported in Fig. 4.8, H_2O_2 and Pb(IV), significantly reduce the percentage of annexin V-FITC/PI double negative MCF-7 cells (viable cells, bottom left panel), and increase the percentage of early apoptotic (annexin V-FITC positive, bottom right panel) and mid-late apoptotic (annexin V-FITC/PI double positive, top right panel) cells.

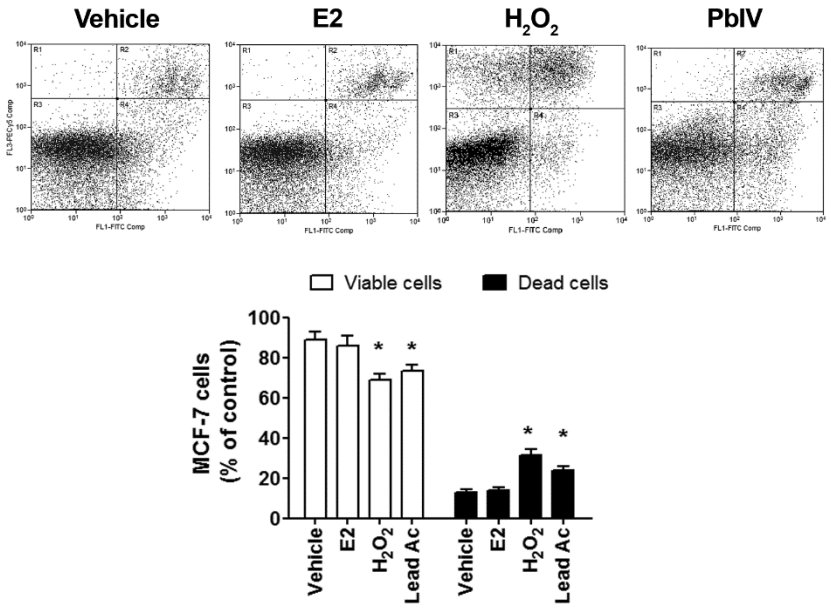

Fig 4.8 Effect of H_2O_2 and Pb(IV) on apoptotic cell death. Typical cytograms of vehicle–, E2- (10 nM), H_2O_2- (400 μM), and Pb(IV)- (200 μM) treated MCF-7 cells for 24 h (top) and relative analyses (bottom) obtained from Annexin V-FITC with PI assays. Data of viable (PI and Annexin V-FITC double negative) and dead (Annexin V-FITC positive and PI AnnexinV-FITC double positive) cells are means ± SD of three different experiments. P< 0.05 was calculated with Student's t test vs vehicle (*).

This result has been further confirmed by Western blot analysis in wild type (ScNGB MCF-7) and stable NGB silenced (ShNGB MCF-7) cells (Fig. 4.9 A). Although both H_2O_2 and Pb(IV) increase NGB level, 24 h after treatment they activate the PARP-1 cleavage, another pro-apoptotic marker, both in the presence and in the absence of NGB (Figs. 4.9 B and C). This result lead us to evaluate if E2-induced NGB over-expression in mitochondria could protect MCF-7 cells from Pb(IV)-induced apoptosis. To reach this aim, MCF-7 cells were stimulated with E2 for 24 h before the Pb(IV) treatment. Fig. 4.9 D clearly indicate that E2

pre-treatment reduces Pb(IV)-induced PARP cleavage in ScNGB MCF-7, while this effect is completely impaired in ShNGB MCF-7 (Fig. 4.9 E).

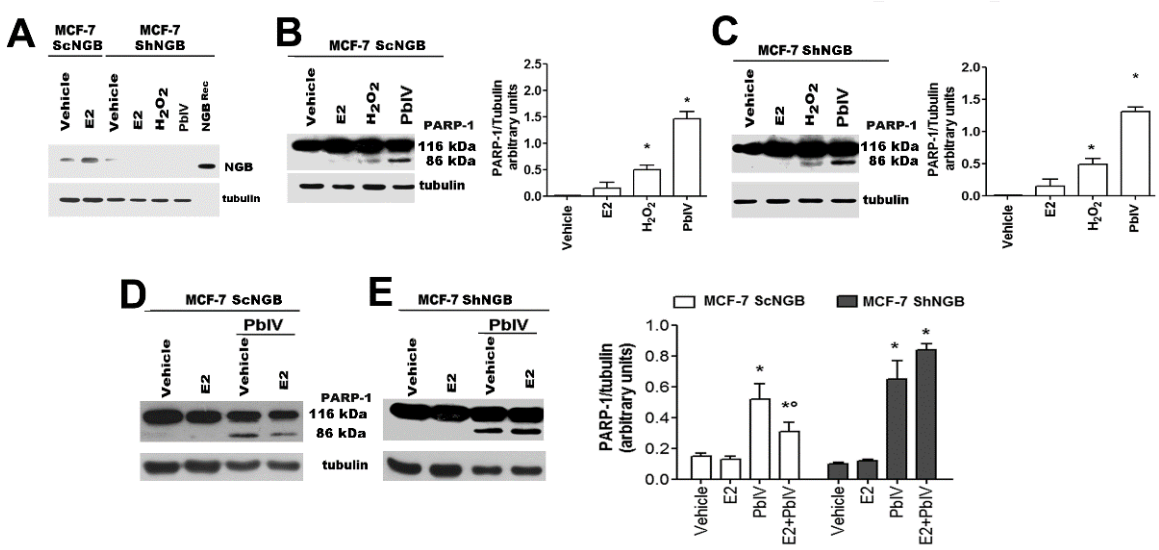

Fig 4.9 Effect of NGB on H_2O_2- and Pb(IV)-induced apoptosis. (A) Western blot analysis of NGB protein levels performed in vehicle- and E2 (10 nM)- treated control MCF7 cells (ScNGB) and NGB stable silenced cells (ShNGB) treated with selected compounds for 24 h. Typical Western blot representative of three independent experiments. **(B)** Western blot analyses of PARP-1 cleavage in MCF-7 cells infected with scramble RNA (ScNGB MCF-7) treated with above reported compounds for 24 h. **(C)** Analysis of protein PARP-1 cleavage in MCF-7 cells infected with silencing NGB shRNA (ShNGB MCF-7) and incubated with E2 (10 nM), H_2O_2 (400 μM), and Pb(IV) (200 μM) for 24 h. Western blot analyses of PARP-1 cleavage in MCF-7 cells infected with scramble RNA (ScNGB MCF-7, **(D)** or with silencing NGB shRNA (ShNGB MCF-7, **(E)** and treated with E2 (10 nM, 24 h) before the treatment with Pb(IV) (200 μM, 24 h). **(B-E)**, Left panels are typical Western blots of three independent experiments. Right panels represent the result of densitometric analyses. The amount of proteins was normalized by comparison with tubulin levels. Data are means ± SD of three different experiments. $P<0.05$ was determined with Student t-test vs. vehicle (*) and Pb(IV) (°).

All together, these results indicate that low NGB level into mitochondria does not protect against apoptosis induced by ROS-inducing compounds.

4.2.7 Effect of Paclitaxel in MCF-7 cell line

Among chemotherapeutic agents used against breast cancer, paclitaxel, a member of the taxane class of antineoplastic agents, has resulted in an improved pathological response when compared with single-agent taxane for the treatment of breast cancer (Buzdar et al., 1999; Luck and Roche, 2002; Sledge et al., 2003). Paclitaxel promotes the formation of unusually stable microtubules, thereby suppressing their dynamics, which lead to the mitotic arrest and apoptosis in dividing cells (Ahn et al., 2004; Gornstein and Schwarz, 2014). However, not all breast tumors are sensitive to paclitaxel. For example, paclitaxel with or without anthracycline (another chemotherapeutic agent) is more effective in ERα negative (24%) than in ERα

positive (11%) patients (Conforti et al., 2007); moreover, ERα knockdown in MCF-7 breast cancer cells increases cell sensitivity to paclitaxel (Tokuda et al., 2012). All together, these data indicate that the ERα expression is an important predictor of the breast cancer response to chemotherapy. On the other hand, the evidence that E2 interferes significantly with paclitaxel antineoplastic activity only when ERα is active both in breast cancer cell (Sui et al., 2007) and in xenograft models (Chang et al., 2012) enlarges the complexity of this picture. Therefore, although the agreement on the low sensitivity of ERα positive breast cancer to paclitaxel treatment is widespread, the role of ERα level or activation status as well as the possible mechanism(s) by which ERα triggers the insensitivity to paclitaxel is still elusive.

Here, the role of the ERα activation status and the involvement of the E2/ERα-dependent up-regulation of NGB in the reduced sensitivity of MCF-7 cells to paclitaxel-induced apoptosis is explored.

Four hours after vehicle and paclitaxel treatment, the pERα level in MCF-7 cells is scarcely detectable, and therefore, the pERα/ERα ratio is very low (Fig. 4.10). As expected (La Rosa et al., 2012), 4 h after E2 stimulation, pERα level increases with the parallel decrease of un-phosphorylated ERα level, and consequently, the pERα/ERα ratio is higher with respect to the vehicle- and paclitaxel-treated MCF-7 cells sustaining that only E2 stimulation enhances the activation status of the receptor (Fig. 4.10).

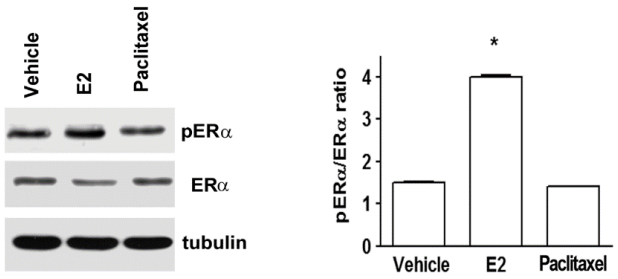

Figure 4.10 Paclitaxel effect on ERα phosphorylation on Ser118. The phosphorylation of the Ser118 residue of ERα (pERα) was determined by Western blot in MCF-7 cells exposed (4 h) to either vehicle (ethanol/PBS 1/10) or E2 (10 nM) or paclitaxel (100 nM). The nitrocellulose was stripped and then probed with anti-ERα and anti-tubulin antibodies. Left panel represents a typical Western blot of three different experiments; right panel represents the pERα/ERα ratio obtained by densitometric analyses of three different experiments (mean ± SD). Significant differences ($P<0.001$) were determined by Student's t-test with respect to vehicle (*).

In MCF-7 cells containing inactivated ERα, paclitaxel treatment increases PARP-1 cleavage in a dose-dependent manner (Fig. 4.11).

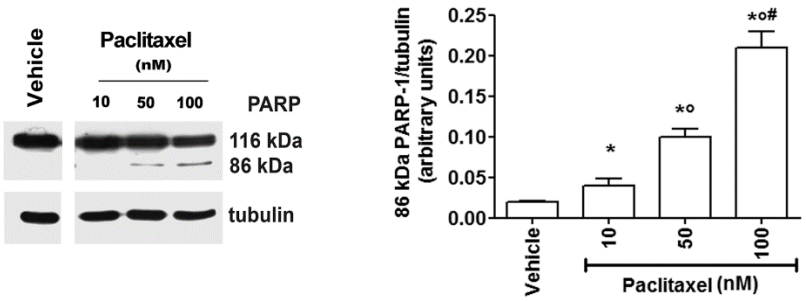

Figure 4.11 Paclitaxel effect on PARP-1 cleavage. Western blot (left) and densitometric analyses (right) of PARP-1 cleavage in MCF-7 cells treated for 24 h with different paclitaxel concentrations. Data are the mean ± SD of five different experiments. P<0.001 was calculated with ANOVA followed by Tukey-Kramer post-test with respect to the vehicle (*) or 10 nM paclitaxel-treated (°) or 50 nM paclitaxel treated (#) samples.

Moreover, paclitaxel significantly reduces the percentage of Annexin V-FITC/PI double-negative cells (viable cells, bottom left boxes) and increases the percentage of early apoptotic (Annexin V-FITC-positive, bottom right boxes) and mid-late apoptotic (Annexin V-FITC/PI double-positive, top right boxes) cells (Fig. 4.12 A). E2 pre-treatment completely impairs the paclitaxel effect increasing the percentage of viable cells (Fig. 4.12 A). This result has been confirmed by the Western blot analysis of PARP-1 cleavage. Although paclitaxel increases PARP-1 cleavage in MCF-7 cells containing inactivated ERα, E2 pre-treatment significantly prevents paclitaxel effect reducing the level of cleaved PARP-1 (i.e., 86-kDa band; Fig. 4.12 B).

Recent evidence supports a role for GPER in E2 action in breast cancer (Filardo et al., 2007). To evaluate that an active ERα is requested by E2 to reduce the paclitaxel-induced apoptosis in breast cancer cells, the E2 effect was assessed in SK-BR-3 cell lines that do not express whole-length ERα, but express high level of GPER (as showed in Figs. 3.2 D and E). Fig. 4.12 C shows that paclitaxel increases PARP-1 cleavage in SK-BR-3 cells even after E2 pretreatment.

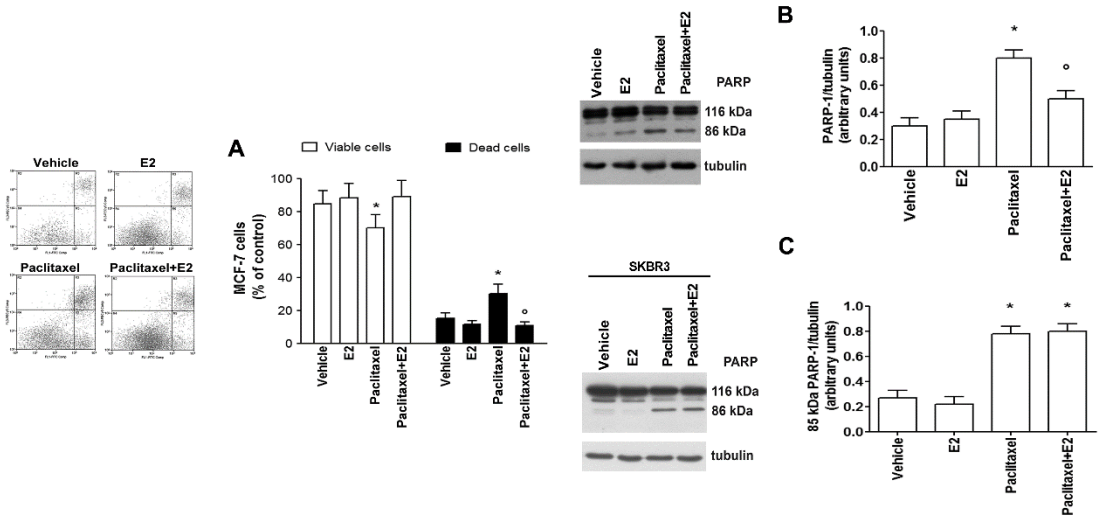

Figure 4.12 E2 impairs paclitaxel-induced apoptosis in the presence of active ERα. (A) Typical cytograms of Annexin assays in vehicle-, E2- (10 nM), paclitaxel- (100 nM), and E2 pretreatment (4 h) + paclitaxel (100 nM)-treated MCF-7 cells for 24 h (left) and relative analyses (right). Viable cells (Annexin V-FITC/PI double negative) are present in the bottom left boxes, early apoptotic cells (Annexin V-FITC-positive) are present in the bottom right boxes, and mid-late apoptotic cells (Annexin V-FITC/PI double-positive) are present in the top right boxes. Data are the mean ± SD of three different experiments. Significant differences ($P<0.05$) were calculated with Student's t-test with respect to either vehicle (*) or paclitaxel-treated (°) samples. Western blot (left) and densitometric analyses (right) of PARP-1 cleavage in MCF-7 **(B)** and SKBR3 **(C)** cells treated with the above reported compounds for 24 h. Data are the mean ± SD of five different experiments. $P<0.001$ was calculated with Student's t-test with respect to the vehicle (*) or paclitaxel-treated (°) samples.

All together these results support literature data, which asserted that E2 interferes significantly with paclitaxel antineoplastic activity only when ERα is active both in breast cancer cell (Sui et al., 2007) and in xenograft models (Chang et al., 2012).

4.2.8 Effects of Paclitaxel on NGB levels

Data shown in Fig. 4.13 A indicate that the ERα activation is necessary to increase NGB levels in MCF-7 cells (Fiocchetti et al., 2014), as demonstrated by the ability of the ERα agonist PPT (10 nM, 24 h) to mimic the E2 effect. Although paclitaxel does not modify ERα levels and phosphorylation (see Fig. 4.10), this compound significantly reduces by half the NGB expression both at the mRNA (Fig. 4.13 B) and at protein (Figs. 4.13 C and D) levels. Intriguingly, the effect of paclitaxel on NGB expression is completely impaired when ERα is active, as demonstrated by the high level of NGB in cells pretreated with E2 before paclitaxel treatment (Figs. 4.13 C and D). To appraise directly the NGB involvement in the E2-induced paclitaxel insensitivity, paclitaxel-induced PARP-1 cleavage has been evaluated in control (ScRNA MCF-7) and NGB stably silenced (shNGB MCF-7) cells.

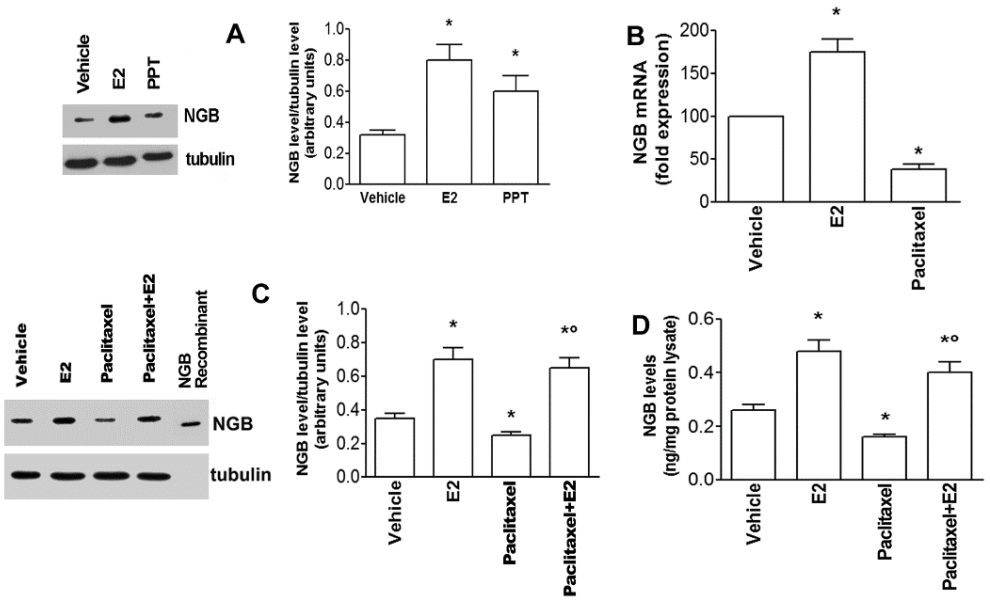

Figure 4.13 E2 and paclitaxel effect on NGB expression. (A) Western blot (left) and densitometric analyses (right) of NGB protein levels in MCF-7 cells treated for 4 h with the vehicle (ethanol/PBS 1/10), E2 (10 nM), and PPT (ERα agonist, 10 nM). Data are the mean ± SD of five different experiments. P<0.001 was determined with Student's t-test with respect to the vehicle (*). **(B)** NGB mRNA levels in MCF-7 cells treated either with the vehicle (ethanol/PBS 1/10) or E2 (10 nM) or paclitaxel (100 nM). The NGB expression is reported as fold of induction over the vehicle (set to 100). Data represent the mean ± SD of five different experiments. P<0.001 was determined by Student's t-test with respect to the vehicle (*). **(C)** Western blot (left) and densitometric analyses (right) of NGB protein levels in MCF-7 cells pretreated 24 h with either the vehicle or E2 (10 nM) or paclitaxel (100 nM) or E2 (4 h) and paclitaxel (100 nM). The amount of protein was normalized to tubulin levels. Data are mean ± SD of three different experiments. P<0.001 was determined with Student's t-test with respect of either the vehicle (*) or paclitaxel-treated (°) samples. **(D)** NGB protein amount (ng/mg protein lysate) in MCF-7 cells treated as reported in panel C. The cell content of NGB protein was quantified by comparing the intensity of the NGB band of Western blot of treated samples with the intensity of the NGB band of 5 ng of the recombinant protein used as the NGB standard. Data are mean ± SD of three independent experiments. P<0.05 was determined with Student's t-test with respect to the vehicle (*) or paclitaxel-treated (°) samples.

As shown in Fig. 4.14 A, NGB is barely detectable in shNGB cells and neither E2 nor paclitaxel modify the protein level. As reported in wild-type MCF-7 cells (see Fig. 4.12 B), 24 h of E2 pre-treatment reduces paclitaxel-induced PARP-1 cleavage in ScRNA MCF-7 (Fig. 4.14 B, upper panels), whereas this effect is completely impaired in ShNGB MCF-7 (Fig. 4.14 B, bottom panels).

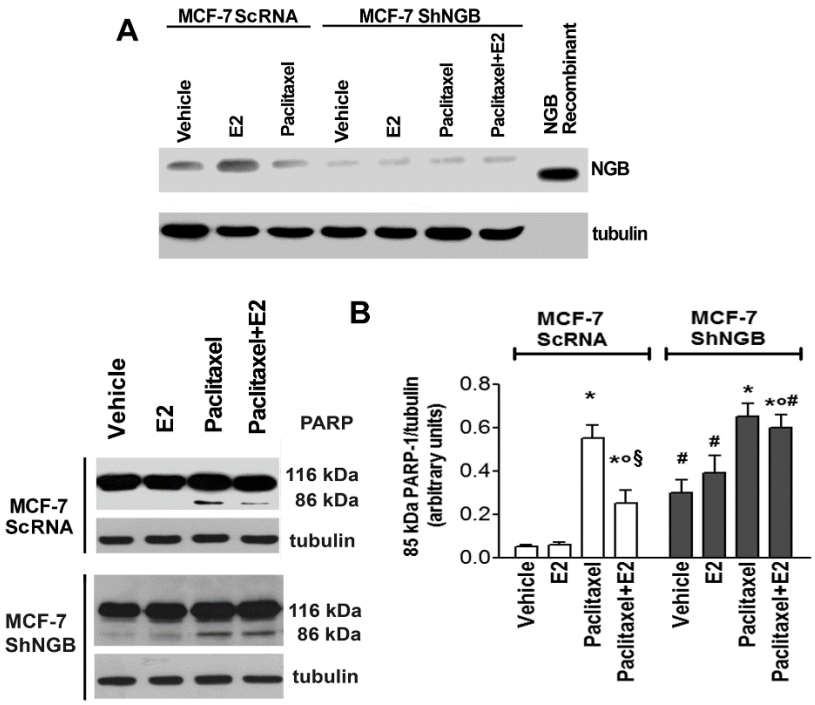

Figure 4.14 NGB involvement in paclitaxel-induced apoptosis. (A) Typical Western blot of NGB protein levels in MCF7 cells infected with control RNA (ScRNA) or with NGB RNA (stable silenced cells, ShNGB) treated 24 h with either the vehicle or E2 (10 nM) or paclitaxel (100 nM) or E2 (4 h) and paclitaxel (100 nM). **(B)** Western blot (left) and densitometric analyses (right) of PARP-1 cleavage in MCF7 cells infected with control RNA (ScRNA) or with NGB RNA (stable silenced cells, ShNGB) treated 24 h as reported in panel A. In all Western blot analyses, the amount of proteins was normalized by comparison with tubulin levels. Densitometric data are mean ± SD of three different experiments. P<0.001 was determined with ANOVA followed by Tukey-Kramer post-test versus either the respective vehicle (*) or E2-treated (°) or paclitaxel-treated (§) samples or versus respective ScRNA-treated cells (#).

Recently, we reported that knockdown of NGB reduces the ability of E2 to increase in MCF-7 cells the level of Bcl-2, the ERα-sensitive anti-apoptotic member of Bcl-2 family, impairing E2-induced anti-apoptotic effects (Fiocchetti et al., 2014). This prompted us to evaluate the modulation of the Bcl-2 level by paclitaxel. As shown in Fig. 4.15, the NGB expression is pivotal to enhance the Bcl-2 level with the hormone effect completely impaired in ShNGB MCF-7 cells. Although paclitaxel reduces by half the NGB level (see Fig. 4.13 C), this chemotherapeutic agent does not affect the Bcl-2 level neither in cells expressing NGB (i.e., ScRNA) nor in ShNGB cells.

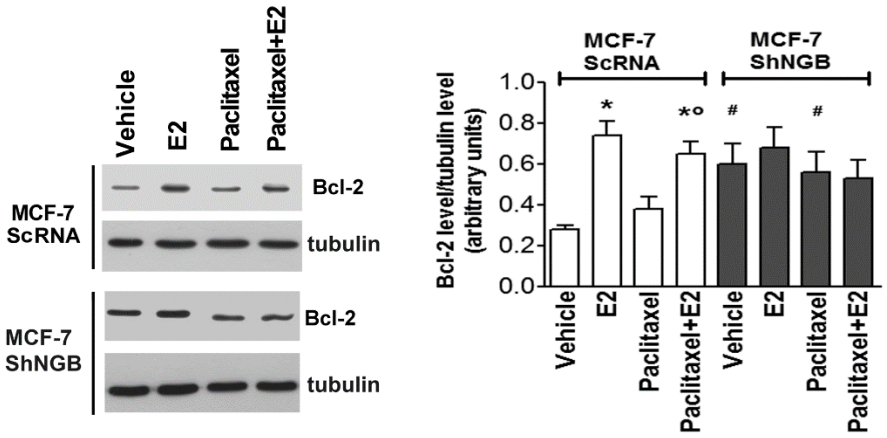

Figure 4.15 Paclitaxel effect on Bcl-2. Western blot (left) and densitometric analyses (right) of Bcl-2 in MCF7 cells infected with control RNA (ScRNA) or with NGB RNA (stable silenced cells, ShNGB) treated for 24 h with either the vehicle or E2 (10 nM) or paclitaxel (100 nM) or E2 (4 h) and paclitaxel (100 nM). The amount of proteins was normalized by comparison with tubulin levels. Densitometric data are mean ± SD of three different experiments. P<0.001 was determined with ANOVA followed by Tukey-Kramer post-test versus either the respective vehicle (*) or E2-treated (°) or paclitaxel-treated (§) samples or versus respective ScRNA-treated cells (#).

4.3 Discussion

In this part of the thesis, we investigated the possible role of high level of NGB in breast cancer cells as a stress sensor and as a compensatory protein, which responds to the injuring stimuli inhibiting the trigger of mitochondria-dependent apoptosis. For this reason, we used different stress conditions (hypoxia, nutrient deprivation, oxidative stress and chemotherapeutic agent) and analyzed the effects on NGB protein levels. Hypoxia is a common feature of solid tumors and the involvement of NGB in the short term adaptation of cancer cells has been hypothesized (Emara et al., 2010). In fact, NGB co-localizes with the hypoxia-inducible metallo-enzyme carbonic anhydrase IX in different human primary tumor specimens (Emara et al., 2010). On the other hand, the hypoxia-dependent up-regulation of NGB mRNA has been assessed in lung cancer cells even if no information is available on the protein level (Oleksiewicz et al., 2011). Although NGB is not transcriptionally regulated by HIF1α the major intracellular oxygen sensor (Jin et al., 2012), this globin has been proposed to be a member of the hypoxia-inducible protein family (Greenberg et al., 2008). Despite this evidence, our results indicate that 2% O2, which resembles the median pO2 present in breast cancer microenvironment (Vaupel et al., 2003), does not up-regulate NGB levels in MCF-7 cells suggesting that NGB is not required for MCF-7 cells adaptation to hypoxic conditions. Of note, myoglobin, which is strongly up-regulated by hypoxia in MCF-7 cells (Gorr et al., 2011), may attend to this function thus suggesting a cell context dependent modulation of

NGB from hypoxia. Nutrient deprivation frequently occur in living organisms and at cellular level under both physiological or pathological situations such as fasting, ischemia or solid tumor development (Caro-Maldonado and Muoz-Pinedo, 2011). Differently from hypoxia, nutrient deprivation up-regulates NGB levels in MCF-7 cells, suggesting a role as compensatory protein of globin. Nutrient deprivation drives cells to autophagy in order to remove damaged organelles and molecules. Thus, this datum open the possibility that besides its role in preventing apoptosis, NGB up-regulation may be involved in the autophagic flux increasing the survival of breast cancer cells. As hypoxia, oxidative stress is characteristic of tumor development. High ROS levels, as occurring in fast proliferating tumor tissues (Gorrini et al., 2013), could lead to severe cellular damage and, consequently, to cell death. However, cancer cells establish several mechanisms to counteract the oxidative stress-induced apoptosis and, generally, display an antioxidant capacity higher than that of normal cells (Gorrini et al., 2013). Different intracellular pathways could converge to alter the cellular metabolism and to adapt cancer cells to both intrinsic and extrinsic oxidative stress conditions (Cairns et al., 2011).

However, the question about the possible role of endogenous NGB in non-nervous cancer cells as an oxidative stress sensor remains unsolved. The results obtained clearly demonstrate that in MCF-7 cells, ROS inducers modulate the level of NGB. Indeed, cell treatment with H_2O_2 and Pb(IV) leads to a rapid increase of intracellular ROS production, and up-regulates NGB protein levels. These data prompted us to verify if selected compounds could modify NGB mitochondrial localization. Remarkably, E2-induced NGB up-regulation exerts anti-apoptotic function directly at the mitochondrial compartment by interacting with cytochrome *c* and impairing its release to cytosol and the consequent activation of the intrinsic apoptotic pathway upon oxidative stress injury (Fiocchetti et al., 2013; Fiocchetti et al., 2014). Present data indicate that in MCF-7 cells, only the E2 treatment almost doubles the NGB amount in the mitochondrial fraction, whereas H_2O_2 and Pb(IV), which increase NGB level in the whole cell, do not affect the mitochondrial protein localization. In order to obtain clear evidence on how NGB level could be regulated by H_2O_2 and Pb(IV), we evaluated the effect of lysosomal, proteasomal and translational inhibitor. The data indicate that H_2O_2 and Pb(IV) effect on NGB level seems to be mediated by the inhibition of NGB lysosomal degradation and by the activation of translation as demonstrated by cell pre-treatment with Chloroquine and Cycloheximide. Contrarily, MG-132 does not modulate NGB level, but completely impairs H_2O_2 and Pb(IV) effect in enhancing NGB level. Recently, a role for MG-132 and

Chloroquine in the activation and inhibition, respectively, of autophagy in breast cancer cells has been reported (Bao et al., 2016; Maycotte and Thorburn, 2014) rendering particularly intriguing these results. Indeed, our results suggest that the autophagic process is involved in H_2O_2- and Pb(IV)-NGB accumulation breast cancer cells sustaining the datum obtained with nutrient deprivation. All together these data indicate that ROS-inducing compounds increase NGB protein levels in MCF-7 cancer cells activating specific pathways divergent from those triggered by E2 (Fiocchetti et al., 2017a). Thus, we demonstrated that ROS-inducing compound triggers the PI3K-independent AKT activation, in a different manner than E2. In particular, E2 stimulation of breast cancer cells triggers the PI3K/PDK-dependent AKT Ser473 phosphorylation whereas the ROS-inducing substance rapidly induces a PI3K-independent AKT activation. Consequently, divergent downstream signals and functional outcomes originates. Indeed, the two pathways differently affect the timing of AKT activation being E2 able to rapidly and persistently activate AKT phosphorylation, whereas Pb(IV) induces the rapid but transient AKT activation (Fiocchetti et al., 2017a). Moreover, E2 and Pb(IV) show a similar ability to induce the AKT phosphorylation even if Pb(IV) induces a three-fold higher AKT phosphorylation after 30 min of stimulation (Fiocchetti et al., 2017a).

Finally, the involvement of AKT could converge on ROS inducing pathways to increase NGB levels and, ultimately, to cancer cell survival. However, data reported here unexpectedly indicate that the increased level of NGB induced by H_2O_2 and Pb(IV) is not sufficient to counteract the ability of these substances to induce the apoptotic death in MCF-7. This result has been further confirmed by NGB silencing experiments in which both H_2O_2 and Pb(IV) still activate the PARP-1 cleavage, an apoptotic marker in MCF-7 cells. However, increasing NGB levels by 24 h E2 treatment reduces Pb(IV) activation of PARP-1 cleavage. This result is in line with the E2 protective effect against H_2O_2–induced apoptosis previously reported (Fiocchetti et al., 2015; Fiocchetti et al., 2014) strongly confirming the anti-apoptotic role of NGB. Although both pathways culminate in NGB up-regulation, E2, but not Pb(IV), stimulation lead to the increased level of NGB into mitochondria strongly sustaining that a persistent activation of AKT is required to modify NGB cell localization. These differences could drive cells to different outcome.

All together, these data indicate that the increase of intracellular NGB levels induced by H_2O_2 and Pb(IV) is not sufficient to reset the intrinsic apoptotic pathway, which requires the re-allocation of NGB into mitochondria (Fig. 4.16).

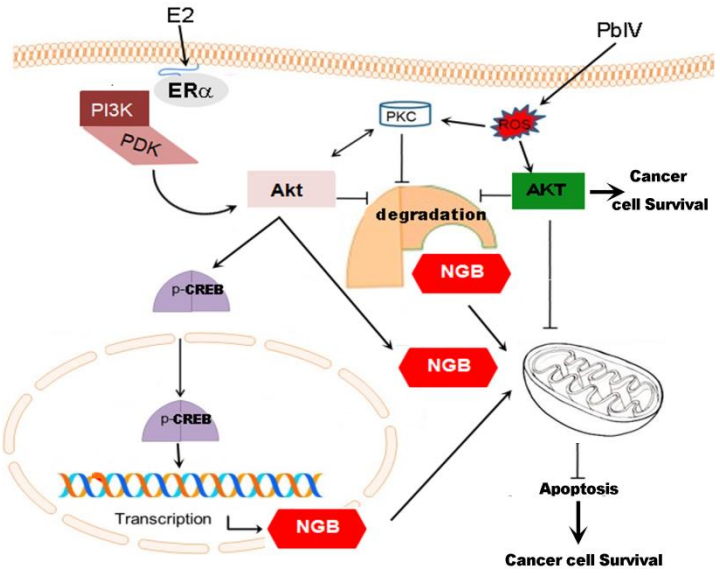

Figure 4.16 Graphic representation of pathways activated by E2 and ROS-inducing compounds that affect NGB compartmentalization into mitochondria.

In line with this idea, Yu and coworkers (Yu et al., 2012a) demonstrated that in primary mouse cortical neurons NGB mitochondrial localization increased after pathological oxygen and glucose deprivation conditions conferring neuroprotection. Although it could be possible that only the high NGB levels obtained by transfection with NGB-encoding plasmid allow the NGB recruitment on lipid rafts reported in neurons after oxidative stress (Watanabe et al., 2012), the possibility that E2, oxygen-glucose deprivation, and oxidative stress signaling could induce different NGB conformation that affect the protein translocation into mitochondria should be taken into account. An opposite function has been attributed to NGB in hepatocarcinoma cells. In this cell line, NGB acts as a mediator between oxygen/ROS signals and the cytosolic signaling cascade that regulates the cell proliferation (Zhang et al., 2013b). Unfortunately, also this evidence has been obtained in cells transiently transfected with the NGB-encoding plasmid which induces high non-physiological protein levels that could be necessary for NGB involvement in intracellular signaling cascade (Zhang et al., 2013b). However, the involvement of NGB in cancer cell proliferation has not been confirmed by MCF-7 cell growth curves, which result similar to the control even when NGB was stably silenced (Fiocchetti et al., 2014). These results indicate that the over-expression of NGB does not fully represent the physiological behavior of NGB.

Furthermore, conversely to the mitochondria-dependent apoptosis induced by H_2O_2 and Pb(IV) (De Marinis et al., 2010; Kahlert et al., 2000), the other compound selected, Paclitaxel

is a potent anti-microtubule agent currently used in the treatment of breast cancers. Paclitaxel stabilizes microtubule and suppresses their dynamics leading to the mitotic arrest and apoptosis in dividing cells *via* a caspase-independent mechanism (Ahn et al., 2004; Gornstein and Schwarz, 2014). As discussed previously, paclitaxel is less effective in presence of active ERα. It is well recognized that ERα activities should be preceded by receptor phosphorylation at the Ser118 residue (pERα; refs. (Ali et al., 1993; La Rosa et al., 2012)). Paclitaxel treatment does not modify pERα level in MCF-7 cells, while, as expected (La Rosa et al., 2012), E2 activates its receptor. These evidences prompted us to evaluate if the active ERα could impair paclitaxel-induced MCF-7 cell death. In MCF-7 cells containing inactivated ERα, paclitaxel treatment induces an increase of apoptotic death; in fact, it induces the cleavage of pro-apoptotic marker, PARP-1, and the percentage of early and mid-late apoptotic cells. E2 pre-treatment completely impairs the paclitaxel effect increasing the percentage of viable cells and decreasing the PARP-1 cleavage.

Recent evidence supports a role for GPER in E2 action in breast cancer (Filardo et al., 2007). To evaluate if an active ERα is requested by E2 to reduce the paclitaxel-induced apoptosis in breast cancer cells, the E2 effect was assessed in SK-BR-3 cell lines that do not express whole-length ERα, but express high level of GPER (Filardo et al., 2007). Our data shows that paclitaxel increases PARP-1 cleavage in SK-BR-3 cells even after E2 pre-treatment. As a whole, these data indicate that the low sensitivity of MCF-7 cells to paclitaxel-induced apoptosis appears to be related to the phosphorylated status of ERα rather than to its level. It has been reported that the stable transfection of ERα results in reduced sensitivity of the BCap37 cells, an ERα negative breast cancer cell line, to paclitaxel-induced apoptotic cell death, without affecting paclitaxel-induced microtubule bundling or mitotic arrest (Sui et al., 2007). Our results are in line with these data indicating that the reduction of MCF-7 cells, endogenously containing ERα, is mainly due to the E2-induced ERα anti-apoptotic activity. Data obtained show that ERα activation is necessary to increase NGB levels in MCF-7 cells (Fiocchetti et al., 2014), as demonstrated by the ability of the ERα agonist PPT to mimic the E2 effect. Although paclitaxel does not modify ERα levels and activity, it significantly reduces by an half of the NGB expression both at the mRNA and at protein levels. Intriguingly, the effect of paclitaxel on NGB expression is completely impaired when ERα is active, as demonstrated by the high level of NGB in cells pretreated with E2 before paclitaxel treatment. To appraise directly the NGB involvement in the E2-induced paclitaxel insensitivity, paclitaxel-induced PARP-1 cleavage has been evaluated in control (ScRNA MCF-7) and

NGB stably silenced (shNGB MCF-7) cells. Data obtained show that NGB is barely detectable in shNGB cells and neither E2 nor paclitaxel modify its level. As described previously, in wild-type MCF-7 cells, the E2 pre-treatment reduces paclitaxel-induced PARP-1 cleavage in ScRNA MCF-7, whereas this effect is completely impaired in ShNGB MCF-7; this finding highlights the involvement of E2/ERα-dependent NGB up-regulation in the insensitivity of MCF-7 to paclitaxel. Recently, we reported that knockdown of NGB reduces the ability of E2 to increase in MCF-7 cells the level of Bcl-2, the ERα-sensitive anti-apoptotic member of Bcl-2 family, impairing E2-induced anti-apoptotic effects (Fiocchetti et al., 2014). This prompted us to evaluate the modulation of the Bcl-2 level by paclitaxel. Our data show that NGB expression is pivotal to enhance the Bcl-2 level with the hormone effect completely impaired in ShNGB MCF-7 cells. Although paclitaxel reduces by half the NGB level, this chemotherapeutic agent does not affect the Bcl-2 level neither in cells expressing NGB (i.e., ScRNA) nor in ShNGB cells. The overexpression of Bcl-2, like that induced by E2, inhibits apoptosis through the inactivation of Bax/Bak (a pro-apoptotic member of the Bcl-2 family), the modulation of the outer mitochondrial membrane permeability, and the inactivation of p53-induced apoptosis (Sasi et al., 2009).

All together, these results support the role of NGB as a compensatory protein in breast cancer cells and they suggest that E2-induced NGB up-regulation could represent a defense mechanism of E2-related human breast cancer that renders cancer cells insensitive to several injury including chemotherapy.

5. PLANT-DERIVED POLYPHENOLS AS NGB LEVEL MODULATORS

5.1 Introduction

As reported in previously chapters, NGB is an E2-inducible and anti-apoptotic protein. In particular, the hormone up-regulates and reallocates NGB into mitochondria of neurons *via* ERβ (De Marinis et al., 2010; De Marinis et al., 2013b) and of breast cancer cells *via* ERα (see chapter 3). In chapter 4, we demonstrated that NGB level could be considered as a sensor of ROS being up-regulated by ROS (i.e., H_2O_2) and by ROS-inducing substances (i.e., Pb(IV)). Moreover, in the absence of active ERα, the chemotherapeutic drug paclitaxel, significantly reduces NGB cell content and induces apoptotic cell death, while in in presence of active ERα its effects on apoptotic cell death decrease. All together, these results suggest that the E2-induced NGB up-regulation in cancer cells could represent a defense mechanism of E2-related human breast cancer rendering them insensitive to several injury including chemotherapy. These results led us to hypothesize that reducing NGB levels in breast cancer could render these cells more prone to stressors. A possible strategy to verify this hypothesis is to individuate a molecule able to antagonize ERα/NGB pathway. However, this molecule should also preserve the ERβ/NGB pathway in neuron-derived cells to maintain the well-known protective effects of NGB in the brain (De Marinis et al., 2010; De Marinis et al., 2013b; Fiocchetti et al., 2012; Fiocchetti et al., 2013).

Plant-derived polyphenols are good candidate to verify our hypothesis. Indeed, plants produce polyphenols as secondary metabolites against environmental stresses. Plant-derived polyphenols possess many biological activities in humans, such as antioxidant, antifungal and antibiotic properties (Lecomte et al., 2017). There is a growing body of evidence that the consumption of some these plants or their polyphenols could be an additive efficient tool to prevent and to treat several dysfunctions and diseases related to aging, mental processes, metabolism, malignant transformation, cardiovascular diseases, breast and prostate cancers, menopausal symptoms, osteoporosis, atherosclerosis and stroke, and neurodegeneration (Branca and Lorenzetti, 2005; Cassidy, 2003; Tuohy, 2003). Based on their chemical structure and in respect to biosynthesis patterns, plant-derived polyphenols may be divided in chalcones, flavonoids (flavones, flavonols, flavanones, isoflavonoids), lignans, stilbenoids,

and miscellaneous classes (Sirotkin and Harrath, 2014). All of these compounds contain one or several aromatic rings with at last one hydroxyl group. Hydroxyl groups can be free, but most of the time they are engaged in another function with an ester, ether or a glycoside (Lecomte et al., 2017). These types of natural compounds show a close resemblance with chemical structure to 17β-estradiol; therefore, they are able to bind ERα and ERβ (Paterni et al., 2014; Rietjens et al., 2013; Younes and Honma, 2011). Polyphenols can act either as estrogen agonists or as antagonists since they may trigger an estrogenic response, thereby binding and activating the ERs, which ultimately induces an estrogenic effect or an antiestrogenic response, blocking or altering the receptor activation by endogenous estrogens and thereby inhibiting an estrogenic effect (Michel et al., 2013). Thus, polyphenols can act as estrogen agonists and antagonists at the same time usually in an organ-dependent manner resulting in mixed agonistic/antagonistic properties (selectivity) depending on the receptor content of specific tissues (ERα and ERβ populations) and the concentration of the endogenous estrogens (Michel et al., 2013).

Here, E2/ERα/PI3K/AKT pathway as a platform for the breast cancer treatment has been used. In particular, we studied the possibility that selective ERα ligands could modify NGB expression. For this reason, we analyzed the ability of different plant-derived polyphenols to interfere with E2 action, in order to prevent E2-induced NGB up-regulation and render breast cancer cells more prone to death.

5.2 Results

5.2.1 Natural compounds effects on NGB level

In order to find a selective ligand of ERs that could modify NGB expression, we used five polyphenols: Quercetin (Que), 8-prenyl-Naringenin (8-prenyl-Nar), Genistein (Gen), Naringenin (Nar) and Resveratrol (Res). The experiments were carried out for 24 h in MCF-7 cells, which expresses the higher concentration of ERα in respect to the other ERα positive cell lines previously considered (see Figs. 3.2 A and B). All compounds were tested at three different concentrations: 0.1, 1, and 10 µM; in particular, 1 µM represents the concentration of polyphenols bioavailable in plasma after a meal rich of these compounds (Scalbert and Williamson, 2000). Divergent results are obtained for each polyphenol. In particular, Que, which is a flavonol contained in apples, onions and other vegetables, does not affect NGB level at 0.1 and 1 µM, but up-regulates NGB protein amount at 10 µM. The concomitant

administration of Que and E2 up-regulates NGB protein amount in the same manner of E2 treatment (Fig. 5.1 A). 8-prenyl-naringenin, which is a prenylflavonoid detect in hops, induces both in the presence and the absence of E2 an increase of NGB levels at all concentration considered. In particular, the concomitant administration of 8-prenyl-Nar and E2 does not further increase NGB level (Fig. 5.1 B), indicating that E2 and 8-prenyl-Nar use the same pathway to promote NGB expression. Gen, which is an isoflavone contained in soy, does not modulate NGB levels at all concentration considered but impairs E2 effect in enhancing NGB level (Fig. 5.1 C).

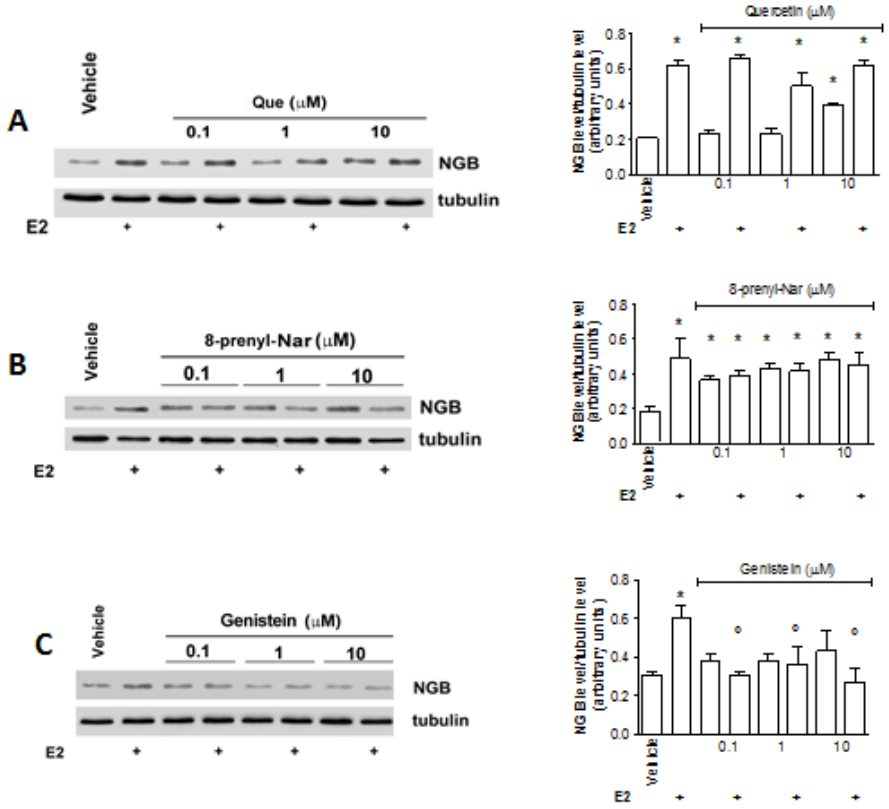

Figure 5.1 Que, 8-prenyl-Nar and Gen effects on NGB level. Western blot (right) and densitometric analyses (left) of NGB protein levels in MCF-7 cells treated for 24 h with the vehicle (ethanol/PBS 1/10), E2 (10 nM), **(A)** with or without Que (0.1, 1, 10 μM), **(B)** 8-prenyl-Nar (0.1, 1, 10 μM), or **(C)** Gen (0.1, 1, 10 μM). The amount of protein was normalized by comparison with tubulin levels. Data are the mean ± SD of five different experiments. P<0.001 was determined with Student's t-test with respect to the vehicle (*) samples or E2-treated (°) samples.

In Figs. 5.2 A and A' the result of Nar stimulation is reported. This compound is a flavanone contained in grapefruit and in a variety of fruit and herbs. Nar does not affect NGB level at all concentration considered (Fig. 5.2 A) but reduces the E2 effect in enhancing NGB level at 0.1 μM after 4 h of treatment (Fig. 5.2 A'). Finally, the effect of Res, a stilbenoid contained in red wine, grape skin and peanuts, on NGB level has been studied. Interestingly, Res down-

regulates NGB levels at 1 and 10 µM but not at 0.1 µM (Fig. 5.2 B). Moreover, it interferes with E2 affect in enhancing NGB level.

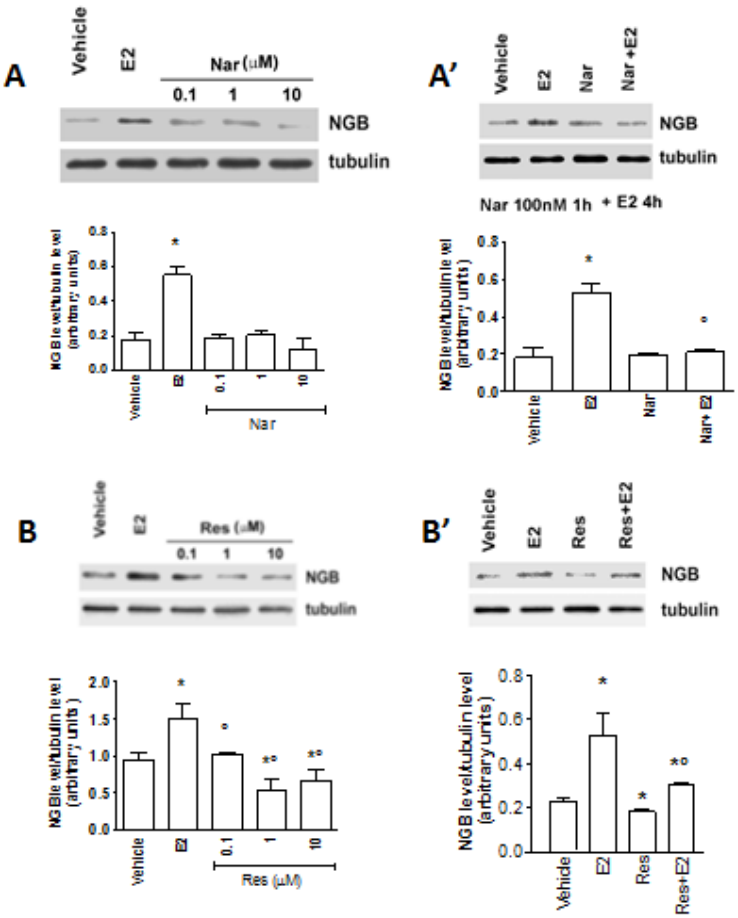

Figure 5.2 Naringenin and Resveratrol effects on NGB level. Western blot (top) and densitometric analyses (bottom) of NGB protein levels in MCF-7 cells treated with the vehicle (ethanol/PBS 1/10), E2 (10 nM), **(A)** Nar (0.1, 1, 10 µM, 24 h), **(A')** with or without Nar (0.1 µM, 4 h), **(B)** with Res (0.1, 1, 10 µM, 24 h), **(B')** with or without Res (1 µM, 24 h). The amount of protein was normalized by comparison with tubulin levels. Data are the mean ± SD of five different experiments. P<0.001 was determined with Student's t-test with respect to the vehicle (*) or E2-treated (°) samples.

As a whole, these data indicate that Res down-regulates globin level; whereas Gen and Nar, just act as E2 antagonists. As our research group works since many years in dissecting Nar action mechanisms (Bulzomi et al., 2012; Galluzzo et al., 2008; Totta et al., 2004) we decided to select Res and Nar for the successive experiments. Intriguingly, both Nar and Res acted at concentration representative of their bioavailability in plasma after a meal rich of these compound (i.e., 1 µM).

To confirm these data, the effects of Nar and Res on NGB level in other ERα positive (T47D) or ERs negative (MDA-MB-231) breast cancer cells have been evaluated. T47D express ERα at much lower concentration in respect to MCF-7 cells (see Figs. 3.2 A and B). In this cell

line, Res impairs E2 effect in enhancing NGB level (Fig. 5.3 A) as shown in MCF-7 cells, but it does not affect NGB expression (Fig. 5.3 A), differently to MCF-7 cells. Instead, as in MCF-7 cells, Nar does not modulate NGB level (Fig. 5.3 B) but impairs E2 action in up-regulating NGB level at 1 and 10 µM, but not at 0.1 µM (Fig. 5.3 B), conversely to MCF-7 cells. Probably, the different results obtained in MCF-7 and T47D cells are due to the dissimilar amount of ERα expressed in them.

Respectively, Fig. 5.3 C and Fig. 5.3 D showed Res (0.1, 1, 10 µM) and Nar (0.1, 1, 10 µM) actions in ERα negative cell line, MDA-MB-231 (see Figs. 3.2 A and B). As expected, none of two compounds affected NGB expression in both presence and absence of E2.

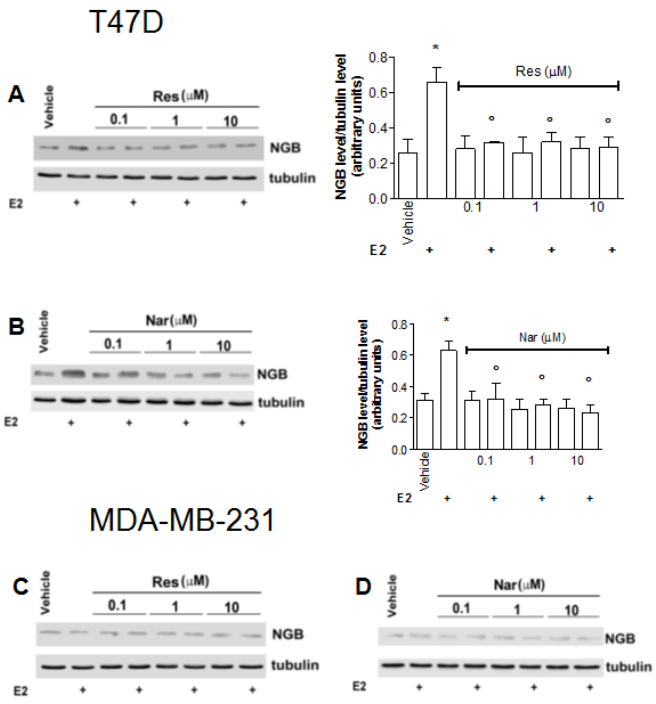

Figure 5.3 Res and Nar effects on NGB level in T47D and MDA-MB-231. Western blot (left) and densitometric analyses (right) of NGB protein levels in T47D cells treated for 24 h with the vehicle (ethanol/PBS 1/10), E2 (10 nM), **(A)** with or without Res (0.1, 1, 10 µM), **(B)** with or without Nar (0.1, 1, 10 µM). The amount of protein was normalized by comparison with tubulin levels. Data are the mean ± SD of five different experiments. P<0.001 was determined with Student's t-test with respect to the vehicle (*) or E2-treated (°) samples. Typical western blot of NGB protein levels in MDA-MB-231 cells treated for 24 h with the vehicle (ethanol/PBS 1/10), E2 (10 nM), **(C)** with or without Res (0.1, 1, 10 µM), **(D)** with or without Nar (0.1, 1, 10 µM). The amount of protein was normalized by comparison with tubulin levels. Data are the mean ± SD of five different experiments.

Finally, in order to evaluated Res and Nar actions in neuron-derived cells, SK-N-BE cells have been used. As showed in Fig. 5.4 A, SK-N-BE express mainly ERβ, whereas ERα is barely detectable. In this cell line, Res (1 µM) strongly up-regulates NGB level and it is not able to interfere with E2 action (Fig. 5.4 B). In Fig. 5.4 C, Nar effect is reported; in particular, the compound does not modified NGB expression at concentration of 0.01 µM after 24 h of

treatment, but at concentration of 0.1 µM up-regulates NGB level, less than E2. Instead, at 1 and 10 µM Nar induces an up-regulation of NGB level comparable with E2 effect.

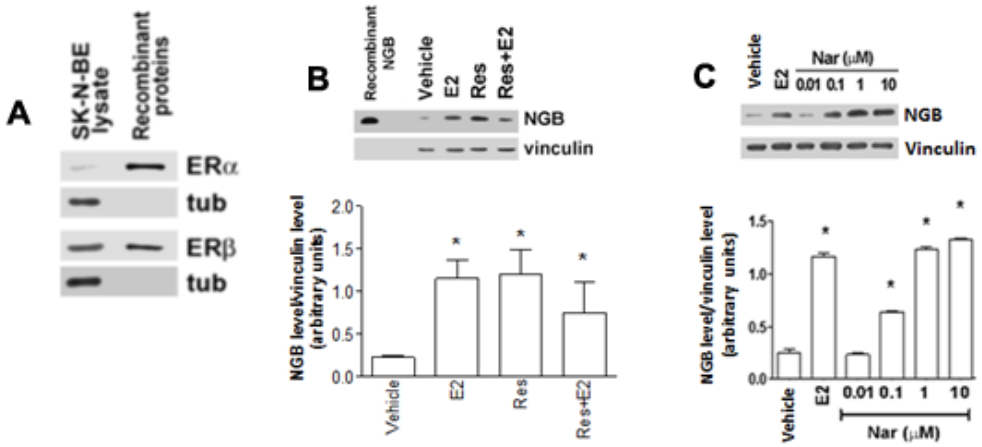

Figure 5.4 Res and Nar effects on NGB protein levels in SK-N-BE. (A) Western blot of ERα and ERβ expression in SK-N-BE. Western blot analyses (top) and densitometric analyses (bottom) of NGB protein levels in SK-N-BE cells treated for 24 h with the vehicle (ethanol/PBS 1/10), E2 (1 nM), **(B)** with or without Res (1 µM), **(C)** and Nar (0.01, 0.1, 1, 10 µM). The amount of protein was normalized by comparison with vinculin levels. Data are the mean ± SD of five different experiments. P<0.001 was determined with Student's t-test with respect to the vehicle (*).

As a whole, these results confirmed that the selected polyphenols act as ERα antagonist and ERβ agonist.

5.2.2 Res and Nar action mechanisms

To further confirm that Res effects in reducing NGB level require ERα, MCF-7 cells were pre-treated with the ERα inhibitor Endoxifen (Endo), a metabolite of Tamoxifen (a selective estrogen receptor modulator).

The MCF-7 cells pre-treatment with Endo (1 µM, 30 min) completely prevents Res (Fig. 5.5) effects on NGB level, confirming that its actions are mediated by ERα.

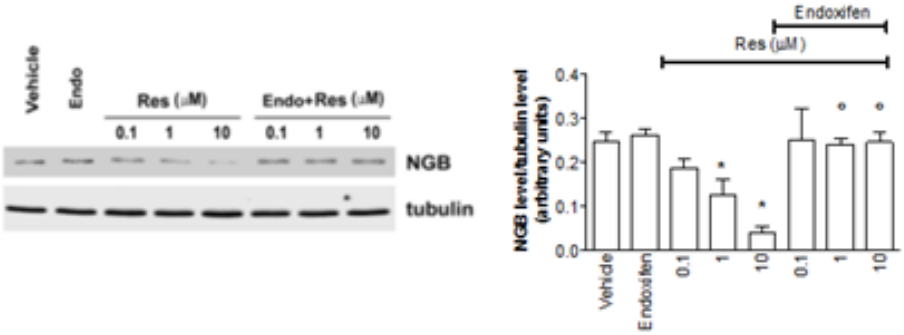

Figure 5.5 ERα mediates Res effects on NGB protein levels. Western blot (left) and densitometry analyses (right) of NGB protein levels in MCF-7 cells treated for 24 h with the vehicle (ethanol/PBS 1/10), Endo (1µM, 30 min pre-treatment) in the

presence or absence of Res (0.1, 1, 10 µM). The amount of protein was normalized by comparison with tubulin levels. Data are the mean ± SD of five different experiments. P<0.001 was determined with Student's t-test with respect to the vehicle (*) or Res-treated (°) samples.

Successively, we evaluated the effects of Nar and Res on the pathways important for NGB up-regulation, i.e., ERα and AKT activation. The results reported in Fig. 5.6 showed that, as expected, E2 activates ERα phosphorylation in Ser118 (pERα) from 30 to 120 min. Nar alone does not induce the ERα phosphorylation and it does not interfere with E2 action on ERα levels and phosphorylation, in line with our previous results (Galluzzo et al., 2008). In parallel, the hormone reduces ERα levels maintaining high level of phosphorylated receptor (Fig. 5.6). On the contrary, Res alone does not induce ERα phosphorylation but, interestingly, it impairs E2 effect in enhancing pERα activation.

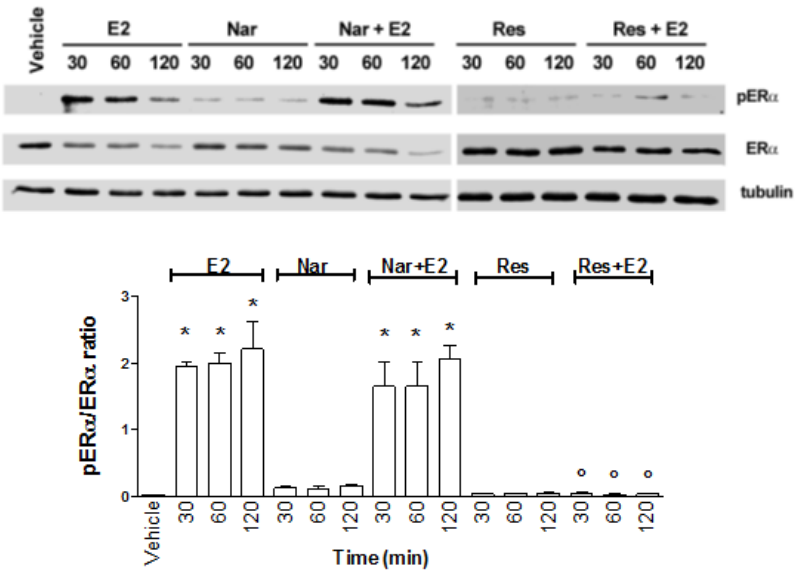

Figure 5.6 Res and Nar effects on ERα activation. The phosphorylation of the Ser118 residue of ERα (pERα) was determined by Western blot in MCF-7 cells exposed (30, 60, 120 min) to either vehicle (ethanol/PBS 1/10) or E2 (10 nM) or in presence or absence of Nar (1 µM) or in presence or absence of Res (1 µM). The nitrocellulose was stripped and then probed with anti-ERα and anti-b-tubulin antibodies. Top panel represents a typical Western blot of three different experiments; bottom panel represents the pERα/ERα ratio calculated with respect to tubulin obtained by densitometric analyses of three different experiments (mean ± SD). Significant differences (P<0.001) were determined by Student's t-test with respect to vehicle (*) or E2-treated (°) samples.

As reported in chapter 4 (see Figs. 4.6 E and F), E2 increases AKT phosphorylation in Ser473 already 30 min after the hormone stimulation and this kinase is pivotal for E2/ERα-induced NGB up-regulation. Both Nar and Res treatments do not induce AKT phosphorylation but they interfere with E2-induced pAKT activation (Fig. 5.7).

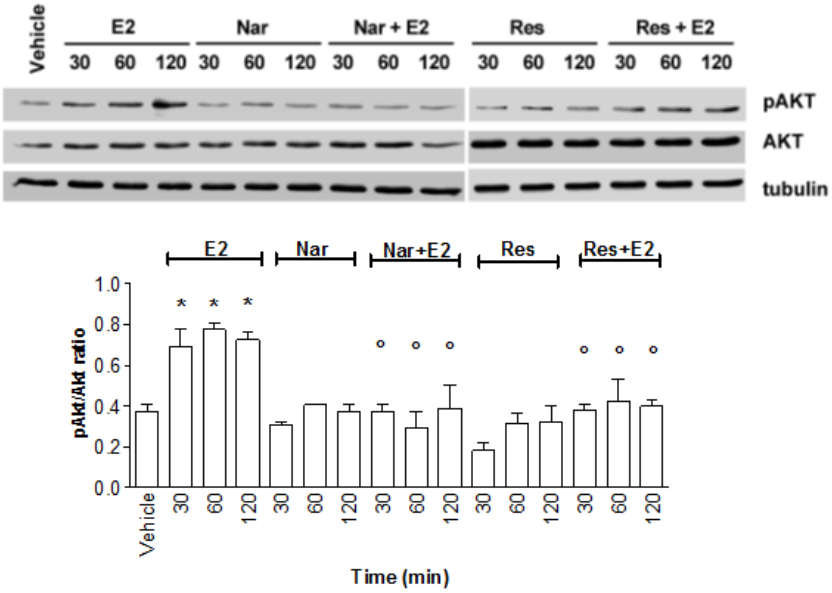

Figure 5.7 Res and Nar effects on AKT activation. The phosphorylation of Ser473 residue of AKT (pAKT) was determined by Western blot in MCF-7 cells exposed (30, 60, 120 min) to either vehicle (ethanol/PBS 1/10) or E2 (10 nM) or in presence or absence of Nar (1 µM) or in presence or absence of Res (1 µM). The nitrocellulose was stripped and then probed with anti-AKT and anti-b-tubulin antibodies. Top panel represents a typical Western blot of three different experiments; bottom panel represents the pAKT/AKT ratio calculate with respect to tubulin obtained by densitometric analyses of three different experiments (mean ± SD). Significant differences ($P<0.001$) were determined by Student's t-test with respect to vehicle (*) or E2-treated (°) samples.

As a whole, these results suggest that Nar effects on decreasing E2-induced NGB levels depends on the impairment of ERα-mediated AKT phosphorylation, while, Res acts through both the inhibition of ERα phosphorylation and AKT activation.

5.2.3 Res and Nar enhance paclitaxel effects on cell death in MCF-7 cells

The results, reported in the previous chapter, demonstrated that as E2/ERα-dependent NGB up-regulation is involved in the insensitivity of MCF-7 to paclitaxel. Here, exploiting the Nar and Res effects on E2-activated pathway *via* ERα and, therefore, on NGB expression, the same compounds action on paclitaxel-induced PARP-1 cleavage has been evaluated.

In Fig. 5.8 A is reported the cellular DNA content after treatment with E2 (10 nM, 48 h), Res (0.1, 1 and 10 µM, 4 h before paclitaxel stimulation) and paclitaxel (100 nM, 48 h). Propidium iodide (PI) assay that determines the PI fluorescence intensity is directly proportional to number of live cells. The results reported in Fig. 5.8 A show that E2 treatment increases cellular DNA content, confirming its proliferative effects. Moreover, as expected by results obtained in previously chapter, paclitaxel decreased significantly the cellular DNA content.

No effect of Res alone effect is detected at 0.1 and 1 µM, while at 10 µM it decreased the cellular DNA content. In contrast, Res enhances paclitaxel effect on cellular DNA content at all concentrations considered. Consequently, the Res effect on paclitaxel-induced PARP-1 cleavage has been performed. The polyphenol (1 µM) alone does not affect PARP-1 cleavage (Fig. 5.8 B), while, as reported in previously chapter (see Fig. 4.12 B), paclitaxel induces the PARP-1 cleavage when ERα is inactivated, but E2 pre-treatment significantly prevents paclitaxel effect reducing the level of cleaved PARP-1. Interestingly, Res pre-treatment enhances paclitaxel effect on PARP-1 cleavage both in presence and in absence of E2 (Fig. 5.8 B).

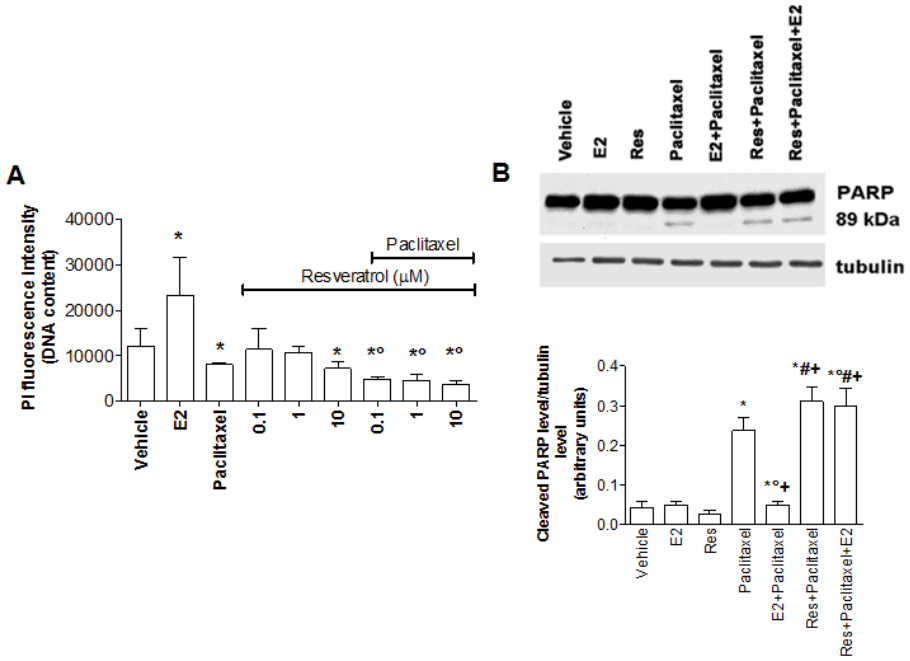

Figure 5.8 Res effects on cellular DNA content and PARP-1 cleavage. (A) Analyses of cellular DNA content obtained from PI assay. The MCF-7 cells are treated for 48 h with vehicle (ethanol/PBS 1/10), E2 (10 nM, 48 h), Res (0.1, 1 and 10 µM, 4 h pre-treatment before paclitaxel stimulation), paclitaxel (100 nM, 48 h). Data are means ± SD of three different experiments. P< 0.05 was calculated with Student's t test vs vehicle (*). **(B)** Western blot (top) and densitometric analyses (bottom) of PARP-1 cleavage in MCF-7 cells treated for 24 h with vehicle (ethanol/PBS 1/10), or E2 (10 nM, pre-treatment 4 h before Paclitaxel stimulation), or in presence or absence of Res (1 µM, pre-treated 1 h before E2 stimulation), or in presence or absence of paclitaxel (100 nM). The amount of protein was normalized by comparison with tubulin levels. Data are the mean ± SD of five different experiments. P<0.001 was determined with ANOVA t-test with respect to the vehicle (*) or E2-treated (°) samples or Paclitaxel-treated (+) samples or Nar-treated (#) samples.

The same experiments are performed using Nar. Fig. 5.9 A shows that Nar does not affect the cellular DNA content at all concentrations considered; instead, it is able to enhance paclitaxel effect on cellular DNA content at 1 and 10 µM but not at 0.1 µM. Fig. 5.9 B shows the Nar effect on paclitaxel-induced PARP-1 cleavage. Nar pre-treatment, as well as Res pre-

treatment, enhances paclitaxel effect on PARP-1 cleavage both in presence and in absence of E2.

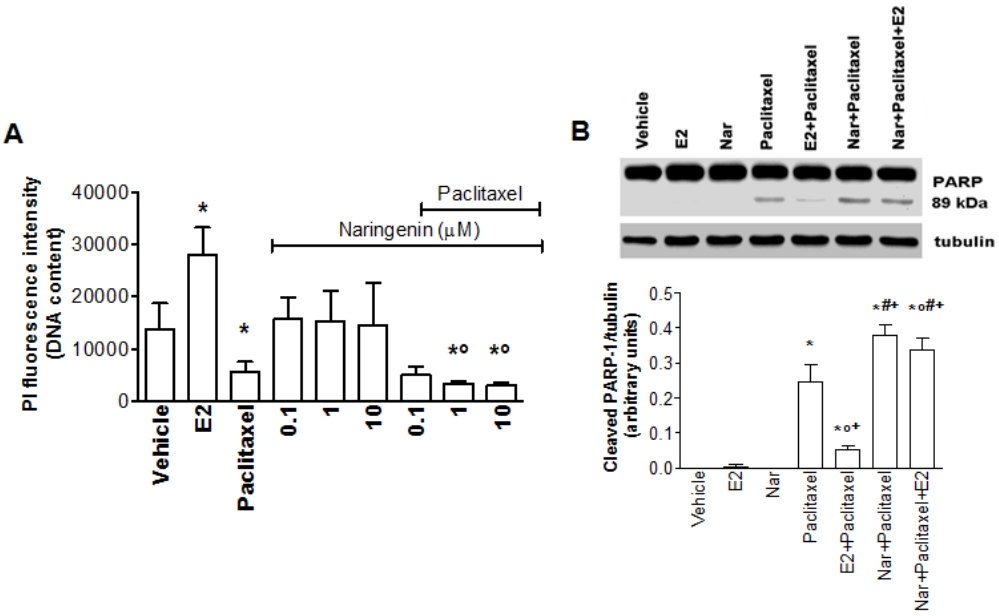

Figure 5.9 Nar effects on cellular DNA content and PARP-1 cleavage. (A) Analyses of cellular DNA content obtained from PI assay. The MCF-7 cells are treated for 48 h with vehicle (ethanol/PBS 1/10), E2 (10nM, 48h), Nar (0.1, 1 and 10 μM, 4 h pre-treatment before paclitaxel stimulation), paclitaxel (100 nM, 48 h). Data are means ± SD of three different experiments. P< 0.05 was calculated with Student's t test vs vehicle (*). **(B)** Western blot (top) and densitometric analyses (bottom) of PARP-1 cleavage in MCF-7 cells treated for 24 h with vehicle (ethanol/PBS 1/10), or E2 (10 nm, pre-treated 4 h before Paclitaxel stimulation), or in presence or absence of Nar (1 μM, pre-treated 1 h before E2 stimulation), or in presence or absence of paclitaxel (100 nM). The amount of protein was normalized by comparison with tubulin levels. Data are the mean ± SD of five different experiments. P<0.001 was determined with ANOVA t-test with respect to the vehicle (*) or E2-treated (°) samples or Paclitaxel-treated (+) samples or Nar-treated (#) samples.

5.3 Discussion

In this part of the thesis, we investigated the ability of plant-derived polyphenols to interfere with E2-activated pathway *via* ERα, in order to prevent NGB over-expression. In literature, data about the effects of plant-derived polyphenols on NGB level are absent. In particular, only Liu and colleagues (Liu et al., 2016) screened a pool of natural compounds on NGB up-regulation in the nervous system. They identified different compounds that can up-regulate both mouse and human NGB promoter activity, and confirmed their data in primary neurons with RT-PCR analyses. Here, we tested the effects of different polyphenols, which are able to bind ERs, on NGB level. Data obtained show different results for each compounds considered. Que, does not affect NGB level at physiological concentration, while, it up-regulates NGB level at 10 μM. Moreover, it does not affect E2-induced NGB up-regulation. 8-Prenyl-Nar up-regulates NGB level, like E2, without enhancing E2 effect on NGB,

suggesting that it is an E2 mimetic. Moreover, the data confirm that the prenylflavonoid acts as an agonist of ERα (Galluzzo and Marino, 2006). Gen does not affect NGB level but it interfere with E2 action on NGB up-regulation. As Gen, Nar does not modify NGB basal level but interfere with E2 action on NGB. Results obtained indicate that both Gen and Nar act as ERα antagonist (Galluzzo and Marino, 2006). Interesting results are obtained with Res. Among the screened compounds, Res is the only one able to decrease NGB levels, moreover, as Nar and Gen, it interferes with E2-induced NGB up-regulation, indicating that Res acts as an ERα antagonist (Nguyen et al., 2017).

After this initial screening, we selected Nar and Res especially because they interfere with basal and E2-stimulated NGB level. Firstly, Nar and Res effects are confirmed in T47D. However, in these cells, Res does not down-regulate NGB level. This discrepancy in the results is probably due to the different ERα expression in each cell line (Fiocchetti et al., 2017a). On the contrary, as expected, in ERα negative cell line, MDA-MB-231, neither Nar nor Res affect NGB levels. In SK-N-BE cells, which expresses in major amount ERβ in respect to ERα, both compounds induce NGB up-regulation, confirming that these polyphenols can act as estrogen agonists and antagonists at the same time depending on the receptor content of specific tissues (ERα and ERβ populations) (Michel et al., 2013). To further confirm the ERα involvement in Nar and Res actions, the cells were pre-treated with the ERα inhibitor, Endoxifen, which is a metabolite of Tamoxifen. Endoxifen is a potent antiestrogen that functions in part by targeting ERα for degradation by the proteasome in breast cancer cells (Wu et al., 2009). Consequently, we evaluated the phosphorylated status of ERα in Ser118 and ATK in Ser473 after Nar and Res stimulation. Our results show that Nar does not induce the ERα phosphorylation and it does not interfere with E2-induced ERα activation. In line with the role of partial antagonist of this substance that impairs only the rapid signals of ERα without affect ERα transcriptional activity (Galluzzo et al., 2008), intriguingly, Nar treatment does not induce AKT phosphorylation but interfere with E2-induced AKT activation, suggesting an antagonistic role of Nar on ERα rapid activities (Galluzzo et al., 2008). Res does not induce ERα phosphorylation but, interestingly, impairs E2-induced ERα phosphorylation; in addition, as well Nar, Res does not activates AKT and interferes with E2-induced AKT phosphorylation, suggesting that Res acts as a complete inhibitor of ERα activity. Thus, these results show that both Nar and Res impair AKT phosphorylation, important for NGB up-regulation.

In the previous chapter, the paclitaxel effect on NGB level and PARP-1 cleavage has been discussed. The data reported showed that paclitaxel does not modify ERα levels and activity (see Fig. 4.10), but it reduces by half the NGB expression both at the RNA and protein levels (see Figs. 4.13 C and D). Moreover, the effect of paclitaxel on NGB expression is completely impaired when ERα is active (see Fig. 4.13 C). Finally, we demonstrated that in wild-type MCF-7 cells, the E2 pre-treatment reduces paclitaxel-induced PARP-1 cleavage in ScRNA MCF-7, whereas this effect is completely impaired in ShNGB MCF-7 (see Figs. 4.14 A, B and C). All together these results demonstrated the involvement of E2/ERα-dependent NGB up-regulation in the insensitivity of MCF-7 to paclitaxel. For this reason, we tested the effects of Nar and Res on E2-activated pathway *via* ERα and, therefore NGB expression, in order to verify if plant-derived polyphenols interfering with E2 and, consequently, with NGB up-regulation could render cells more sensitive to chemotherapeutic treatment.

Different studies have shown that Res has a strong chemopreventive effect against the development of cancers of the skin, breast, prostate and lung (Bishayee, 2009; Goswami and Das, 2009; Kundu and Surh, 2008). The evidence for the cancer chemopreventive effect of Res appeared rather convincing, because it was shown to prevent tumorigenesis in a number of animal models (Bishayee, 2009). Another study demonstrated that the co-encapsulation of Res and paclitaxel in nanocarries could generate potent cytotoxicity against the drug-resistant tumor cells in *vitro* and enhance the bioavailability and the tumor-retention of the drugs in *vivo* (Meng et al., 2016). Indeed, Fukui and colleagues reported that Res strongly diminished the susceptibility of MDA-MB-435s, MDA-MB-231 and SK-BR-3 cells to paclitaxel-induced cell death in culture, although this effect was not observed in MCF-7 cells (Fukui et al., 2010). All these experiments were performed with high Res concentration. Our experiments, performed in MCF-7 cells, showed that Res treatment, at concentration compatible with its bioavailability, does not reduce cell viability and does not activate the apoptotic cascade. However, low Res concentration could enhance paclitaxel-dependent redaction of cellular DNA content and activation of apoptotic cascade. More interestingly, Res, in presence of both E2 and paclitaxel, disables E2 action on paclitaxel enhancing PARP-1 cleavage (Cipolletti et al., manuscript in preparation).

Similarly, a previous study revealed that Nar inhibits proliferation and migration of prostate cancer cells (PC3 and LNCaP), while it induces apoptosis and ROS production by those cells; moreover Nar enhances the efficiency of paclitaxel to suppress progression of prostate cancer cell lines (Lim et al., 2017). Here, we demonstrated that, like Res, Nar

enhances paclitaxel effects on cellular DNA content and PARP-cleavage. Moreover, also Nar, in the presence of both E2 and paclitaxel, disables E2 action on paclitaxel enhancing PARP-1 cleavage.

The results reported here confirm our hypothesis that plant-derived polyphenols are able to prevent E2 -induced NGB up-regulation and could render cancer cell more prone to chemotherapeutic agents.

6. CONCLUDING REMARKS

In 2000, Burmester and colleagues discovered in the nervous system the third heme-globin, called Neuroglobin (NGB) (Burmester et al., 2000). NGB shares Myoglobin (Mb) typical structure: it is a monomer of about 150 amino acids with a molecular weight of 17 kDa (Burmester et al., 2000; Dewilde et al., 2001) and a typical 3-over-3 α-helical sandwich structure. Since its discovery, an increasing number of papers about the role of NGB in the brain has been published.

NGB protective and anti-apoptotic functions in neurons against diverse injuring stimuli (i.e. hypoxia, oxidative stress) are starting to be defined (Antao et al., 2010; Ascenzi et al., 2016; Jin et al., 2008; Liu et al., 2009; Yu et al., 2009; Yu et al., 2012a). The heme-protein structure of NGB led to consider a possible role of the protein also in extra-nervous cancers, which are commonly exposed to hypoxic and oxidative stresses (Gorrini et al., 2013). Although breast cancer is a remarkably heterogeneous disease, subsets of tumors show recurrent compensatory mechanisms of adaptation whose understanding could be critical to improving treatment/management of the disease (Neve et al., 2006).

In the literature, contradictory data about the presence and the role of NGB in cancer have been reported. In particular, Emara and colleagues reported that NGB is expressed in several nervous and non-nervous cancer and in their normal counterparts (Emara et al., 2010). On the other hand, Gorr and colleagues did not find significant levels of NGB transcript could be detect in non-nervous tumors and normal organs (Gorr et al., 2011). Moreover, a role as tumor suppressor has been reported in NGB-overexpressing hepatocarcinoma cells where the globin acts as a linkage between O_2/ROS signals and intracellular signalling that is important for cell proliferation (Zhang et al., 2013a; Zhang et al., 2013b). Previous works performed in our laboratory indicates that E2 *via* ERβ exerts anti-apoptotic and protective functions in neurons by up-regulating and re-allocating NGB into mitochondria (De Marinis et al., 2010; De Marinis et al., 2013b; Fiocchetti et al., 2012; Fiocchetti et al., 2013). In addition, in hepatocarcinoma and in breast cancer cells, E2 *via* ERα induced the up-regulation and re-localization of NGB into mitochondrial compartment in which the globin counteracts apoptosis induced by oxidative stress without any further effect on E2-induced cell

proliferation, suggesting a specific role of the globin in the apoptotic pathway (Fiocchetti et al., 2014).

As a whole, these data raise the hypothesis that NGB could represent one of the vital mechanisms triggered by E2 to increase the survival of cancer cells in the presence of oxidative stress. Thus, this thesis was aimed to investigate functions and action mechanisms of NGB as an E2-induced compensatory protein in E2-related cancers in order to develop a new therapeutic strategy against E2-related breast cancer. Consequently, the main goals of this thesis were: (i) to dissect the signalling pathways, important for cancer cell survival, which up-stream E2-induced NGB over-expression; (ii) to evidence the function played by NGB in E2-related cancer; (iii) to screen natural compound able to interfere with E2-activated pathway *via* ERα, in order to interfere with NGB over-expression in breast cancer cells.

As the correlation between NGB levels and cancer is still unclear (Emara et al., 2010; Gorr et al., 2011), we firstly evaluated its expression in human breast tissues. The experiments, carried out in ERα positive ductal carcinoma specimens selected from 55-75 years old patients, show the expression of both NGB and ERα activated status in tumoral samples but not in normal ones, confirming a key role of the globin in breast cancer physiology. Moreover, a strong correlation between NGB and ERα is demonstrated. In order to obtain a more precise correlation between NGB level and ERα activation, we considered five breast cancer cell lines, characterized by a different ERα expression. NGB is expressed in all cell lines considered but E2 dependent NGB up-regulation is maintained only in ERα-containing breast cancer cell lines (i.e., MCF-7, T47D, ZR-75-1). On the other hand, no E2 effects were reported in ERs negative cells (i.e., SK-BR-3 and MDA-MB-231) confirming a closer relationship between the expression of ERα and modulation of NGB (Fiocchetti et al., 2017a). Taken together, these data strongly sustained our hypothesis confirming a closed relationship between the activation of ERα and the E2-induced NGB over-expression in breast cancer; moreover the results show that E2/NGB pro-survival pathway represents a conserved activated mechanism in ERα breast cancer. Interesting results are obtained in ERα negative SK-BR-3 cell line, in which high NGB amount is observed but not under E2 regulation suggesting that an active not yet identified signaling pathway might exist in this cell line.

Such results prompted us to define the signaling pathway that controls E2-related NGB expression. Firstly, we demonstrate that both the impairment of protein degradation and the enhancement of protein synthesis are at the root of E2-dependent rapid NGB up-regulation. Moreover, using a library of intracellular protein kinases inhibitors, we demonstrated that both

PI3K/PDK/AKT pathway and PKC activation, an upstream activator of AKT (Urtreger et al., 2012), are required for the E2-induced increase of NGB levels in both MCF-7 and T47D cells. In particular, E2 rapidly and persistently activates AKT phosphorylation and the pathway culminates in NGB up-regulation and re-allocation into mitochondria (Fiocchetti et al., 2017a). The high NGB level in whole cells and mitochondria, paralleled with the high AKT phosphorylation status, found in ERα negative and Her2/Neu positive SK-BR-3 cells, strongly sustains the role of AKT. To further confirm the pivotal role of AKT in NGB accumulation, no activation of AKT and barely detectable level of NGB are reported in the triple negative MDA-MB-231 cell line. Our result indicate the crucial role of transcription factor CREB (one of the AKT downstream transcriptional factors) in the E2-induced modulation of NGB levels in both MCF-7 and T47D cells. Therefore, the rapid and persistent E2/ERα-induced AKT assure the rapid NGB accumulation into the cells, the long-term transcription of *NGB* gene, and the translocation of NGB into mitochondria were NGB exerts its role in promoting cell survival and avoiding the triggers of apoptotic cascade (Fiocchetti et al., 2015; Fiocchetti et al., 2014). Thus, we define NGB as a conserved compensatory protein induced by E2 and identify the ERα activated PI3K/AKT signaling as the main intracellular upstream pathway by which E2 affects NGB levels and mitochondrial compartmentalization (Fiocchetti et al., 2017a).

Consequently, we evaluated the possibility that high NGB level could act in breast cancer cells, like in neurons, as a compensatory protective protein activated in response to injuring stimuli and able to prevent mitochondria-dependent apoptosis. To evaluate this hypothesis we tested the effects of different stressors on the level, localization, and function of NGB in wild-type or NGB stable silenced breast cancer cells. The data indicate that hypoxia does not affect NGB protein amount in breast cancer cells (Fiocchetti et al., 2016b), while nutrient deprivation induces an up-regulation of the globin (Fiocchetti et al., 2017b). Moreover, NGB level could be considered as a sensor of ROS being up-regulated by ROS (i.e., H_2O_2) and by ROS-inducing substances (i.e., Pb(IV)), whereas its function as an anti-apoptotic protein is strictly linked to its level and intracellular localization (Fiocchetti et al., 2016b). However, oxidative conditions increase the heme-Fe-based NGB reactivity by formation of the labile Cys46-Cys55 disulfide bond and of the Tyr44/His64/heme propionate interaction (Morozov et al., 2014; Nicolis et al., 2013). Consequently, ROS and ROS-generating compounds induce high level of oxidized NGB that increase, for example, NGB activity as free radical scavenger (Li et al., 2011) supporting the role of NGB as a compensatory protein in breast cancer cells.

Finally, we demonstrated that the E2-induced NGB up-regulation is involved in the MCF-7 cell insensitivity to paclitaxel-induced apoptosis (Fiocchetti et al., 2016a). Our results indicate that in MCF-7 cells, prototype of ERα-positive breast cancer (Neve et al., 2006), an active ERα is at the root of MCF-7 insensitivity to paclitaxel. Furthermore, our data reveal the pivotal role played by NGB in the E2/ERα-induced anti-apoptotic pathway that abrogates paclitaxel induced cell death. Moreover, in the absence of active ERα, paclitaxel significantly reduces the NGB cell content, suggesting that the NGB down-regulation could represent a mechanism by which chemotherapeutic drugs render cancer cells more prone to death (Fiocchetti et al., 2016a).

Finally, we used the E2/ERα/PI3K/AKT pathway as a platform for the breast cancer treatment, in particular screening different natural compounds able to interfere with E2 actions. The selected compounds showed different effects on NGB and E2-induced NGB up-regulation. In particular, we selected Naringenin (Nar) and Resveratrol (Res), which are able to interfere with E2-induced NGB up-regulation; moreover, while Nar does not affect NGB level, Res decreases globin protein amount. Consequently, using ERα positive, ERs negative and ERs positive cell lines, we showed different effects of selected compounds on NGB and E2-induced NGB up-regulation, confirming that phytoestrogens act as estrogen agonists and antagonists depending on the receptor content of specific tissues (ERα and ERβ populations) (Michel et al., 2013). Thus, we evaluated the Nar and Res effects on ERα and AKT activation, and demonstrated that Nar effects on decreasing E2-induced NGB levels are mediated by the inhibition of E2-induced AKT phosphorylation. Instead, Res acts through the inactivation of ERα phosphorylation (Cipolletti et al., manuscript in preparation). Finally, we demonstrated that both Res and Nar, preventing E2-induced NGB up-regulation, enhance paclitaxel effect on inducing apoptosis cell death rendering cancer cell more prone to death.

Although these data should be confirmed in animal models, the results reported here shed light on promising compound, Nar and Res, able to interfere with E2-induced NGB up-regulation and able to render breast cancer more sensitive to chemotherapeutic treatment.

7. MATERIALS AND METHODS

Reagents. E2, Pen-Strep solution, Dulbecco's modified Eagle medium (DMEM) with/without phenol red, McCoy's 5A medium, RPMI-1640 medium with/without phenol red, charcoal-stripped fetal calf serum, protease inhibitor cocktail, bovine serum albumin fraction V (BSA), phosphatase inhibitor cocktail, L-glutamine, PBS, Tris buffer, SDS, anti-tubulin, anti-vinculin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bradford protein assay was obtained from Bio-Rad Laboratories (Hercules, CA, USA). The anti-phospho-ERα (pERα Ser118), anti-pCREB, anti-phospho-AKT (pAKT Ser473) antibodies was purchased from Cell Signalling Technology Inc. (Beverly, MA, USA). Specific antibodies against, AKT, PR, EGFR, ERα (HC20), Neuroglobin, Bcl-2, PARP-1, GPER, Her2/Neu, CREB, TRAP-1, HIF1α, PP2A, mitochondrial marker COX-IV, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Chemiluminescence reagent for Western blot ECL was obtained from Bio-Rad (Hercules, CA, USA).
All the other products were from Sigma-Aldrich. Analytical or reagent grade products were used without further purification.

Preparation and purification of human recombinant NGB. NGB cDNA was cloned into the pET3a vector (Novagen EMD Biosciences, Inc., Madison, WI, USA). The over expression of NGB was induced in the *Escherichia coli* strain BL21(DE3)pLysS (Invitrogen, Carlsbad, California, USA) by treatment with 0.4 mM of isopropyl-D-thiogalactopyranoside (IPTG) in the presence of the heme-precursor aminolevulinic acid (1 mM). Soluble cell extract was loaded onto a DEAE-Sepharose Fast Flow (GE Healthcare Biosciences, Amersham Biosciences Ltd, UK) anion-exchange column equilibrated with 5 mM Tris-HCl, pH 8.5 and fractions were eluted with a NaCl gradient (from 0 to 300 mM). Eluted NGB was further purified by passage through a Sephacryl S-100 (GE Healthcare Biosciences, Amersham Biosciences Ltd, UK) gel filtration column. The protein obtained was > 98% pure on SDS-PAGE. The NGB concentration was determined spectrophotometrically, acquiring UV-visible spectra on a Cary 300 spectrophotometer (Varian, Palo Alto, CA). Five ng of recombinant NGB (final dilution: 1μg/1μl) were loaded in Western blot and the intensity of the bands was compared by densitometric analyses. Note that, due to recombinant NGB

purification, its migration on SDS PAGE resulted faster than that of NGB present in whole cell lysates.

Cell culture. Human breast cancer cells MCF-7, T47D, MDA-MB-231, ZR-75-1, SK-BR-3 (ATTC, LGC Standards S.r.l., Milano, Italy) were grown in air containing 5% CO_2 in modified, phenol red-free, DMEM or McCoy's 5A (SK-BR-3) medium containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml) and penicillin (100 U/ml). Human neuroblastoma cell line, SK-N-BE (ATCC, LGC Standards S.r.l., Milan, Italy) was routinely grown at 37 °C in air containing 5% CO2 in modified, phenol red-free, RPMI-1640 medium containing 10% (v/v) fetal calf serum, L-glutamine (2.0 mM), and the Pen-Strep solution (penicillin, 100 U/ml; streptomycin, 100 mg/ml). Cells were passaged every 2 days. Cells were used at passage 4-8. Cells were grown to approximately 70% confluence in 6-well plates before stimulation. The cell line authentication was periodically performed by amplification of multiple STR loci by BMR genomics srl (Padova, Italy). NGB stably silenced MCF-7 cells were obtained using lentiviral particles with scramble RNA as control (ScRNA) and NGB RNA (ShNGB) as previously described (19). The lentiviral-infected MCF-7 cell line was routinely grown in media containing puromycin (0.5 lg/mL). Cells were treated for 4 or 24 h with either vehicle (ethanol/PBS 1:10, v/v) or E2 (10 nM) or Pb(IV) (200 μM). When indicated, Wortmanin (Wort; 1 μM), Arachidonic Acid Leelamide (AAL; 1μM), Bisindolymal eimide XI (BIM-IX; 1μM), ML-9 (10 μM), Triciribine (Tric, 1 μM), MG-132 (MG 1 μM), Chloroquine (Chl, 10 μM) or Cycloheximide (Cxe, 10 μM for 30 min) were added 30 min before E2 or Pb(IV) administration.

Hypoxic treatment. MCF-7 cell lines were grown to 70% confluence in 6-well plates and stimulated with either vehicle or E2 (10 nM). After 2h of stimulation, cells were cultured in normoxia using an incubator (KW Apparecchi Scientifici, Siena, Italy) set at 5% CO_2, 21% O_2 (atmospheric oxygen ~140 mmHg), and 37.0°C in a humidified environment. For the experiments under hypoxia, a water-jacketed incubator (Forma Scientific, Marietta, OH, USA) has been used to provide a customized and stable humidified environment through electronic control of CO_2 (5%), O_2, and temperature (37.0°C). The O_2 tension was set and maintained constantly at 2% (~14mmHg) by injecting N_2 automatically in the chamber.

Protein extraction and Western blot assay. Protein extraction and Western blot assay were performed as reported elsewhere (Fiocchetti et al., 2016b). Briefly, after treatment, cells were lysed and solubilized in the sample buffer (SB) containing 0.125 M Tris-HCl, pH 6.8, and

0.75 % (w/v) SDS. Tissue samples were homogenized in 10 vol of SB with 65 strokes in a glass-Teflon homogenizer. Homogenates were pulse (2s on, 1s off) sonicated with 45% of amplitude for 30' and then centrifuged at 10,000× g for 10 min. Supernatant was collected for western blots. Total proteins were quantified using the Bradford Protein Assay. Solubilized proteins (20 µg) were resolved by 7% or 15% SDS-PAGE at 100 V for 1 h at 24.0 °C and then transferred to nitrocellulose with the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA) for 7 min. The nitrocellulose was treated with 3% (w/v) BSA in 138.0 mM NaCl, 25.0 mM Tris, pH 8.0, at 24.0 °C for 1 h and then probed overnight at 4.0 °C with either anti-NGB (final dilution 1:1000) or anti GPER (final dilution 1:1000) or anti-PR (final dilution 1:1000) or anti-EGFR (final dilution 1:5000) or anti-Her2/Neu (final diluition 1:1000) or HIF1α (final dilution 1:1000) or anti-PARP-1 (final dilution 1:1000) or anti-pCREB (final dilution 1:1000) or anti-Bcl-2 (final dilution 1:1000) or anti- TRAP-1 (final dilution 1:1000) or anti-PP2A (final dilution 1:1000) or anti-pERα (final dilution 1:1000) or anti p-AKT (final dilution 1:1000) antibodies. The nitrocellulose was stripped by the Restore Western Blot Stripping Buffer (Pierce Chemical, Rockford, IL, USA) for 30 min at room temperature and then probed with anti-ERα (final dilution 1:1000) or anti-CREB (final dilution 1:1000) or anti AKT (final dilution1:1000)or anti-α-tubulin (final dilution 1:30.000) or anti-vinculin (final dilution 1:30.000) antibodies (final dilution 1:1000) to normalize the protein loaded. When indicated 5.0 ng of recombinant NGB (final dilution: 1µg/1µl) (kindly gifted by Dr. Cinzia Verde) were loaded in Western blot. Note that, due to recombinant NGB purification, its migration on SDS PAGE resulted faster than that of NGB present in whole cell lysates. The antibody reaction was visualized with the chemiluminescence Western blotting detection reagent (Amersham Biosciences, Little Chalfont, UK). The densitometric analyses were performed by the ImageJ software for Microsoft Windows (National Institutes of Health, Bethesda, MD, USA).

Intracellular ROS measurement. Cells were seeded in clear bottom 96-well microplate with 2.5×104 cells per well. After allowing cells to adhere overnight, the medium was removed and cells washed once with serum free medium. Then, cells were incubated with dichloro-dihydro-fluorescein diacetate (DCFH-DA, 20 µM, purchased from Sigma-Aldrich, St. Louis, MO, USA) at 37°C, 30 min in the dark. After this time, the DCFH-DA solution was removed and cells washed once with serum-free medium and treated with selected compounds; background wells (untreated stained cells) as well as blank wells (medium only) were included. The microplates were read in the presence of compounds and media on a multi-label

plate reader (VICTOR™ X3 Multilabel Plate Reader, PerkinElmer, Waltham, MA, USA) with excitation wavelength at 485 nm and emission wavelength at 535 nm to measure fluorescence intensity for each time interval (from 0 to 6 h). The fluorescence was registered as arbitrary units, the ratio between the single treatment induced fluorescence, and the vehicle fluorescence was plotted for each time considered.

Quantitative Real-Time Polymerase Chain Reaction. The sequences for gene-specific forward and reverse primers were designed using the OligoPerfect Designer software program (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA). The following primers were used: for human NGB 5'-GTCTCTCCTCGCCTGAGTTC-3' (forward) and 5'-GACTCACCCACTGTCGAGAA-3' (reverse), and for human GAPDH, 5'-CGAGATCCCTCCAAAATCAA-3' (forward) and 5'-TGTGGTCATGAGTCCTTCCA-3' (reverse). Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Life Technologies Corporation) according to the manufacturer's instructions. To determine NGB gene expression levels, cDNA synthesis and qPCR were performed using the GoTaq two-step RT-qPCR system (Promega, Madison, WI, USA) in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Each sample was tested in triplicate and the experiment was repeated twice. All primers used were optimized for real-time amplification in a standard curve amplification (>98% for each pair of primers) and verifying the production of a single amplicon in a melting curve assay. The results were normalized to the expression of GAPDH mRNA. The relative level of the NGB gene, reported in arbitrary units, was calculated using the 2-ΔΔCt method.

shRNA Lentiviral particles transduction. shRNA lentiviral particle transduction was performed using control shRNA lentiviral particles (Santa Cruz sc-108080) and NGB shRNA lentiviral particles (Santa Cruz sc-42081-v) according to the manufacturer's instructions. Cells were plated in a 12-well plate for 24 h before the viral infection. Cells were grown to ~ 50% confluence and then infected. Growth media were removed and replaced with 1 ml of a mixture of complete medium with Polybrene (Santa Cruz sc-134220) at a final concentration of 5 μg/ml; then, shRNA lentiviral larticles were added to the culture. After 24 h of incubation, the culture medium was removed and replaced with 1 ml of complete medium without Polybrene. After overnight incubation, cells were splited 1 : 3 and maintained in incubation for 24 h in the complete medium. Finally, pooled stable clones expressing the shRNA were

selected by incubating cells with a mixture of complete medium with puromycin. Next, the medium was replaced with fresh puromycin-containing medium every 3 to 4 days until resistant colonies were identified. Colonies were expanded and assayed for stable shRNA expression.

Apoptosis measurement. Phosphatidylserine externalization was quantified by flow cytometry by using the Annexin V-FITC Apoptosis Detection Kit including propidium iodide (PI) according to the manufacturer's guideline (Santa Cruz, CA, USA). Briefly, both attached and floating cells were collected after treatment(s), washed twice with cold PBS and re-suspended in the annexin-binding buffer at a concentration of ~1×106 cells/ml; 100 µl of the cell suspension (~1×105 cells) were transferred to a culture tube and 2.5 µl of annexin V-FITC and 10 µl of PI were added. After incubation in the dark (15 min at room temperature), 400 µl of the binding buffer were added and cells were analyzed immediately by flow cytometry with the DAKO Galaxy flow-cytometer equipped with HBO mercury lamp. Analysis by flow cytometry used the FL1 (FITC) and FL3 (PI) laser lines; each sample was assessed using a collection of 10,000 events. Each experiment was carried out in triplicate and the fluorescence was calculated using a FloMax© Software.

Mitochondria isolation. Cell fractionation was performed using ApoAlert™ Cell Fractionation kit (Clontech Laboratories Inc. Mountain View, CA, USA) according to manufacturer's instructions. After stimulation, cells were harvested with trypsin (1%, v/v), suspended with complete medium, and centrifuged at 600g for 5 min. Pellet was suspended in Fractionation Buffer Mix containing DTT 1 mM and homogenized in a Dounce tissue grinder. Homogenate was centrifuged at 700g for 10 min. Pellet was suspended in Fractionation Buffer Mix to obtain mitochondrial fraction. The mitochondrial TNF-receptor associated protein 1 (TRAP-1) and cytosolic Protein Phosphatase 2A (PP2A) markers were used as mitochondrial fraction purity indicators. Protein concentration of each fraction was determined using Bradford protein assay. Lysate of each fraction was then processed for Western Blot or used for immunoprecipitation assay.

Confocal Microscopy Analysis. MCF-7 and T47D cells were stained with anti-NGB (1:200) and anti-COX-4 (1:200) antibodies, respectively. Cells were processed in chamber slides and rinsed with PBS, pH 7.4, followed by fixation in formaldehyde 4% (v/v) for 1 hour, and permeabilization with cold acetone 95% (v/v) for 3 min. Cells were rinsed in PBS and saturated with BSA 2% (w/v) for 1 hour and then incubated with primary antibody at 4 °C o/n

(anti-NGB) or 1 hour at RT. After that cells were rinsed three times in PBS for 5 min and incubated with Alexa Fluor 488® and 578® donkey anti-mouse secondary antibodies (Invitrogen) (1:400). The slides were cover-slipped using Prolong® Gold anti-fade reagent. Confocal analysis (63× magnification) was performed using LCS (Leica Microsystems, Wetzlar, Germany). The 8.2 IMARIS software was used to quantify NGB-COX-4 merged signals.

Statistical analysis. The statistical analysis was performed by Student's *t*-test to compare two sets of data or with ANOVA followed by Turkey-Kramer post-test to compare more groups of treatments by INSTAT software system for Windows. In all cases, only probability (*P*) values below 0.05 were considered significant.

Cellular DNA content with propidium iodide (PI). MCF-7 cells were grown until 80% confluence in 96 well plate and treated with the selected compounds. The cells were fixed and permeabilized with frozen EtOH 70% for 15 min at -20°C. EtOH soluzion were removed and the cells were incubated with propidium iodide (PI) buffer for 30 min in the dark. The solution were removed and the cells were rinsed with PBS solution. The fluorescence were revealed (Excitement 537 nm, Emission 621 nm) with Spark 20M multimode microplate reader.

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APPENDIX A

ROMA TRE UNIVERSITÀ DEGLI STUDI	**DOTTORATO IN**
	SCIENZE E TECNOLOGIE BIOMEDICHE '

CICLO XXX

Dottoranda Manuela Cipolletti

Anno accademico 2016/2017 Anno di corso 3°

Titolo del progetto di ricerca: Estrogen/Neuroglobin pathway as pharmacological target for hormone-related cancer

REPORT OF THE ACTIVITIES CARRIED ON DURING PhD

ACTIVITIES A.A. 2014-2017	CFU
Seminars	26
Annual Meeting	12
Congress	20
Courses	9
	TOT: 67

Publications:

1. Fiocchetti M, **Cipolletti M,** Leone S, Naldini A, Carraro F, Giordano D, Verde C, Ascenzi P, Marino M. Neuroglobin in breast cancer cells: effect of Hypoxia and oxidative stress on protein level, localization, and anti-apoptotic function. PLoS One 2016, 11(5): 1-14.
2. Fiocchetti M, **Cipolletti M**, Leone S, Ascenzi P, Marino M. Neuroglobin overexpression induced by the 17β-Estradiol-Estrogen receptor-α Pathway reduces the sensitivity of MCF-7 Breast cancer cell to paclitaxel. IUBMB Life 2016, 68(8): 645-51.
3. D'Alonzo D, **Cipolletti M**, Tarantino G, Ziaco M, Pieretti G, Iadonisi A, Palumbo G, Alfano A, Giuliano M, De Rosa M, Schiraldi C, Cammarota M, Parrilli M, Bedini E, Corsaro MM. A Semisynthetic Approach to New Immunoadjuvant Candidates: Site-Selective Chemical Manipulation of Escherichia coli Monophosphoryl Lipid A. Chemistry 2016, 22(31): 11053-63.
4. Fiocchetti M, **Cipolletti M**, Brandi V, Polticelli F, Ascenzi P. Neuroglobin and friends. J Mol Recognit 2017, e2654.
5. Fiocchetti M, Cipolletti M, Marino M. Compensatory role of Neuroglobin in nervous and non nervous cancer cell in response to the nutrient deprivation. PLoS One 2017, 12(12):e0189179.

6. Fiocchetti M, **Cipolletti M**, Ascenzi P, Marino M. Dissecting the 17β-estradiol pathways necessary for Neuroglobin translocation to the mitochondria and anti-apoptotic activity in breast cancer. J cell Physiol.
7. Campesi I, Marino M, **Cipolletti M**, Romani A, Franconi F. Diet-derived polyphenols in a gender perspective. Submitted to Food Chem.
8. **Cipolletti M**, Fiocchetti M, Montalesi E, Nuzzo MT, Marino M. Resveratrol counteracts 17β-estradiol/estrogen receptor α-induced neuroglobin upregulation increasing the susceptibility of breast cancer cells to the apoptotic death. Manuscript in preparation

Oral communications:

Cipolletti M, Fiocchetti M, Nuzzo MT, Ascenzi P, Marino M. Neuroglobin: an estrogen-inducible protein involved in cancer cell survival. 9th "Annual Meeting of Young Researchers in Physiology". Florence, May 7-9, 2015.

Fiocchetti M, **Cipolletti M**, Nuzzo MT, Ascenzi P, Marino M. Neuroglobin: the double-edged sword of 17β-Estradiol against oxidative stress in cancer cells. International Workshop on "Biochemistry, Physiology and Pharmacology of Oxidative Stress". Rome July 2-4, 2015.

Fiocchetti M, **Cipolletti M**, Marino M. NGB translocation to mitochondria: a specific 17β-estradiol anti-apoptotic pathway in hormone dependent breast cancer cells. 67th Congress of the Italian Physiological Society. Catania September 21-23, 2016.

Cipolletti M, Nuzzo MT, Fiocchetti M, Marino M. The effect of the food component Naringenin on Neuroglobin, an E2-inducible protein involved in cell survival. 11th "Annual Meeting of Young Researchers in Physiology". Florence, May 25-27, 2017.

Fiocchetti M, **Cipolletti M**, MarinoM. Responses of nervous and non-nervous cells to nutrient deprivation overexpress compensatory protein: impact on autophagy process. 68th SIF National Congress "Italian Physiology Society". Pavia, September 6-8, 2017.

Poster presentations:

Fiocchetti M, **Cipolletti M**, Nuzzo MT, Ascenzi P, Marino M. 17β-Estradiol/Neuroglobin pathway: a new anti-apoptotic signal for hormone-dependent target cells. 23rd ECDO Conference 'Death pathway and beyond'. Geneve (CH) October, 2015.

Fiocchetti M, **Cipolletti M**, Nuzzo MT, Pallottini V, Acconcia F,. Ascenzi P, Marino M. 17β-Estradiol/Neuroglobin a new pathway against oxidative stress in cancer cells. Congresso Società Italiana Fisiologia. Genoa September, 2015.

Cipolletti M, Fiocchetti M, Marino M. 17β-estradiol-induced neuroglobin up-regulation acts as anti-apoptotic pathways that reduces breast cancer cell sensitivity to chemotherapeutic agents. 67th Congress of the Italian Physiological Society. Catania September 21-23, 2016.

Cipolletti M, Fiocchetti M, Ascenzi P, Marino M. The estrogen-induced anti-apoptotic pathway reduces the sensitivity of breast cancer cell to the chemotherapeutic agent paclitaxel.

24th conference of the European Cell Death Organization, "*Cell Death* in Health and Disease". Barcelona September 28-30, 2016.

Fiocchetti M, **Cipolletti M**, Ascenzi P, Marino M. 17β-estradiol anti-apoptotic pathway rely on NGB translocation to the mitochondria in breast cancer cells. 24th conference of the European Cell Death Organization, "*Cell Death* in Health and Disease". Barcelona September 28-30, 2016.

Fiocchetti M, **Cipolletti M**, Nuzzo MT, Ascenzi P, Marino M. Neuroglobin: a sensor of oxidative stress in breast cancer cell lines. XIXth International conference on "Oxygen binding and sensing proteins". Hamburg September 12-14, 2016.

Cipolletti M, Nuzzo MT, Fiocchetti M, Marino M. Two food components, Naringenin and Resveratrol, regulates estrogen receptors activities involved in cell survival. 68th SIF National Congress "Italian Physiology Society". Pavia, September 6-8, 2017.

APPENDIX B

Evaluation PhD Thesis

All concerns raised by referees were addressed and amended.

Questions:

1. It seems that recNGB migrates to a lower MW!!!

   Yes, the recNGB migrates to a lower MW. This is probably due the fact that the recNGB is a purified protein and also because it is obtained in bacteria in which the proteins do not affect to post translational modification. So the two proteins may not be identical.

2. Why do you expect the non-modulation of NGB levels by the compounds in the absence of E2 in MDA-MB-231?

   In the last part of this thesis, we studied the possibility that polyphenols, interacting with ERα, could modify NGB expression in both presence or absence of E2. I expect the non-modulation of NGB levels in absence of E2 in MDA-MB-231 because this is an ERα(-) cell line.

EXTERNAL EVALUATION REPORT

Candidate (Name, Surname): Manuela Cipolletti

PhD thesis entitled: Estrogen/Neuroglobin pathway as pharmacological target for hormone-related cancer

Reviewer (Name, Surname): Elias Castanas

Institution & Address: University of Crete, School of Medicine, Laboratory of Experimental Endocrinology, P.O. Box 2208, Heraklion, GR-71110, Greece

E-mail: castanas@uoc.gr

A. Specific questions

Query	Answer (yes/no)
The PhD thesis provides an original contribution to current knowledge in the field	Y
Aims of the PhD thesis are clearly identified	Y
The PhD thesis denotes adequate knowledge of the literature in the field	Y
The PhD thesis denotes that the candidate has sufficient methodological and technical skills to conduct the research	Y
Results are clearly illustrated	Y
Results are properly discussed	Y
The style of text and figures is satisfactory	Y
References are exhaustive and updated	Y

B. Comments on the acceptability of the PhD thesis for the final dissertation (free text)

This very well-written thesis explores the role of neuroglobin (NGB), a heme-containing protein, in breast cancer. The candidate clearly demonstrates the modulation of the protein by phosphorylated estrogen receptor alpha and the signaling events leading to NGB modification through rapid ERα-signaling. Meticulously investigating different intracellular pathways, the candidate proposes convincingly the signaling events leading from ERα to NGB. In addition, part of the proposed signaling has been further used as a platform for the investigation of possible natural modulators of NGB and their possible use as novel adjuvant chemotherapeutics in hormone-sensitive breast cancer.

The thesis is well written and the candidate clearly identifies the research questions which are convincingly and clearly illustrated in the manuscript. Part of this work has already been evaluated by peers and published in the international scientific literature, with the candidate's name in a prominent position. In addition, the scientific output of the candidate is very good (7 publications in Medline-indexed journals) and two papers in preparation/review, as derived from the provided text.

DOTTORATO DI RICERCA SCIENZE E TECNOLOGIE BIOMEDICHE

ROMA TRE
UNIVERSITÀ DEGLI STUDI

Coordinatore: Prof. Paolo Visca

In my opinion, and in view of the submitted manuscript, Mrs Manuela Cipolletti has all the necessary credentials for the public presentation of her Thesis, pending some minor corrections/comments I have provided in the annotated manuscript.

Date: Heraklion, December 20, 2017

Signature:

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EXTERNAL EVALUATION REPORT

Candidate (Name, Surname): Manuela Cipolletti

PhD thesis entitled: Estrogen/Neuroglobin pathway as pharmacological target for hormone-related cancer.

Reviewer (Name, Surname): Francesco Grignani

Institution & Address: Department of Medicine, University of Perugia, Italy

E-mail: francesco.grignani@unipg.it

A. Specific questions

Query	Answer (yes/no)
The PhD thesis provides an original contribution to current knowledge in the field (yes/no)	Yes
Aims of the PhD thesis are clearly identified (yes/no)	Yes
The PhD thesis denotes adequate knowledge of the literature in the field (yes/no)	Yes
The PhD thesis denotes that the candidate has sufficient methodological and technical skills to conduct the research (yes/no)	Yes
Results are clearly illustrated (yes/no)	Yes
Results are properly discussed (yes/no)	Yes
The style of text and figures is satisfactory (yes/no)	Yes
References are exhaustive and updated (yes/no)	Yes

B. Comments on the acceptability of the PhD thesis for the final dissertation (free text)

The thesis by Manuela Cipolletti shows an in depth analysis of the role of NGB as a pro-survival molecule in breast cancer cells, possibly identifying a new pathway for cancer cell self-protection from apoptosis. Further experiments identify a number of molecules, which may interfere with the Estrogen-NGB pathway and may be studied for their use in increasing the effects of chemotherapy in breast cancer.
The thesis is clearly written and illustrated. The literature is correctly and extensively cited. The thesis describes a large amount of work, applying several different approaches and

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techniques and generating a number of interesting results. The data are reported in several papers co-authored by Dr. Cipolletti.

Overall, the thesis is fully acceptable for the final dissertation.

Signature

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