University of Algarve

Faculty of Science and Technology

INVESTIGATING THE ROLE OF ANNEXIN A2 IN EPIDERMAL GROWTH FACTOR (EGF) INDUCED SIGNALLING IN CANCER

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Master of Science Thesis

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Work supervised by:

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A Thesis Submitted to the Department of Biomedical Sciences and Medicine of the University of Algarve in Partial Fulfilment of the Requirements for the Award of the Erasmus Mundus Master in Quality in Analytical Laboratories, supervised by Dr Patrícia Alexandra Madureira (Cancer Biology and Progression Laboratory, Centre for Biomedical Research - CBMR)

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Statement of authorship

I hereby declare that I am the author of this work, which is, to the best of my knowledge and belief, original, except as acknowledged in the text. Authors and work consulted are properly cited in the text and listed in the references in the required format. The material has not been previously submitted, in whole or in part, for a degree at this or any other university.

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Signature

DEDICATION

This thesis is dedicated to all children, adolescents and adults battling with different types of cancer worldwide.

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"A journey of a thousand miles begins with a single step"

Laozi (604 - 531 BC)

ABSTRACT

Reactive oxygen species (ROS) are produced as a consequence of cellular metabolism and can also be produced by the cell in response to growth factor/growth factor receptor stimulation to function as second messengers in major signalling pathways. Recently, increasing evidence has revealed that the ROS, H_2O_2 is an important second messenger in cellular signal transduction, because of its high diffusion and ability to selectively target reactive cysteine residues in proteins. Currently, H_2O_2-mediated signalling has been implicated in several fundamental physiological processes such as cell proliferation, differentiation, migration and apoptosis. EGF/EGFR is one of the most mutated GF/GFR associations in cancer thanks to its role in tumorigenesis. The binding of EGF to EGFR induces downstream events leading to intracellular production of H_2O_2 for signalling. Cancer cells characteristically exhibit increased ROS levels compared to normal counterparts that gives them a proliferative advantage and promotes cancer progression. To balance the advantage of low ROS levels (nanomolar concentration for proliferative signalling pathways) against its damaging effect (as a specific oxidant at high concentrations), cancer cells induce the cellular antioxidant response. Our laboratory identified a novel redox regulatory protein, annexin A2 (ANXA2) and showed that its antioxidant function plays a crucial role in supporting tumour growth and chemoresistance. As a logical follow up to this research we investigated the role played by ANXA2 in oncogenic signalling pathways induced by EGF. Here, the activation of signalling pathways in pre-established MDA MB 231 breast cancer cell lines with knockdown for ANXA2 and respective control cells was investigated by western blotting upon treatment of these cells with 50 ng/ml of EGF at different time points. Biotinylated Iodoacetamide (BIAM) assays were done to assess the oxidation of reactive Cys residues in redox sensitive proteins upon treatment with EGF. Intracellular ROS levels and cell proliferation were also analysed in ANXA2 depleted versus control cancer cells upon treatment with EGF. Our results show that ANXA2 depletion in MDA MB 231 cancer cells leads to enhanced activation of the pro-survival and pro-proliferative PI3K/Akt signalling pathway, enhanced ROS production and increased proliferation rate upon EGF treatment compared to control cells. EGF treatment also led to oxidation/inhibition of the main regulator of the PI3K/Akt pathway, PTEN. Interestingly, we observed upregulation of PRDX II (a redox regulatory protein) in ANXA2 depleted MDA MB 231 cells. Taken together, our results demonstrate that ANXA2 plays a redox regulatory role in EGF induced ROS-mediated PI3K/Akt signalling and that ANXA2 knockdown cells might be upregulating PRDX II to compensate for the loss of the ANXA2 redox regulatory protein.

Key words: Annexin A2 (ANXA2), reactive oxygen species (ROS), epidermal growth factor (EGF), phosphoinositide 3-kinase (PI3K)

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List of abbreviations

A:

AIIt – Annexin A2 Heterotetramer

AMPK - 5'-AMP-activated Protein Kinase

ANXA2 – Annexin A2

AP-1 - Activator Protein-1

ARE - Antioxidant Response Elements

ASK 1 - Apoptosis Signal Regulating Kinase 1

ATG4 – Autophagy Related Protein 4

ATP – Adenosine Triphosphate

B:

BCA - Bicinconinic Acid

bFGF - Basic Fibroblast Growth Factor

BIAM – Biotinylated Iodoacetamide

BNIP3 - Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3

BSA – Bovine Serum Albumin

C:

Cul3 – Cullin 3

Cys – Cysteine

D:

DCF – Dichlorofluorescein

DCFH-DA - Dihydrodichlorofluorescein Diacetate

DMEM – Dulbecco's Modified Eagle's Medium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

E:

EDTA - Ethylenediaminetetraacetic Acid

EGF – Epidermal Growth Factor

EGFR (ErbB, HER) - Epidermal Growth Factor Receptor

EGTA - Ethylene Glycol Tetraacetic Acid

ER – Endoplasmic Reticulum

ERK - Extracellular Signal-Regulated Kinase

ETC – Mitochondrial Electron Transport Chain

F:

FBS – Foetal Bovine Serum

FOXO - Forkhead Box Protein O

G:

GDP/GTP - Guanosine 5′-dihosphate /Guanosine 5′-triphosphate

GF/GFR – Growth Factor/Growth Factor Receptor

GPX – Glutathione Peroxidase

GSH – Glutathione

GSSG - Glutathione Disulfide

H:

H_2O_2 – Hydrogen Peroxide

HCl – Hydrochloric Acid

HIF - Hypoxia inducible factor

HRP – Horse Radish Peroxidase

I:

IgG – Immunoglobulin G

IL-1 – Interleukin – 1

Ile - Isoleucine

J:

JNK – c-Jun N-terminal kinase

K:

Keap1 - Kelch-like ECH-associated Protein 1

L:

Leu - Leucine

M:

MAPK - Mitogen-Activated Protein Kinase

MCL-1 - Myeloid Cell Leukemia–1

mRNA – Messenger RNA

mtDNA – Mitochondrial DNA

mtROS – Mitochondrial ROS

MTT - 3-[4, 5-Dimethylthiazol-2-yI]-2, 5-Diphenyltetrazolium Bromide

N:

Na_3VO_4 - Sodium Orthovanadate

NAC - N-acetyl-L-cysteine

NaCl – Sodium Chloride

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NaF – Sodium Fluoride

NES - Annexin A2 Nuclear Export Sequence

NF-κB - Nuclear Factor Kappa-light-chain-enhancer of activated B Cells

NOX – NADPH oxidase

Nrf2 - Nuclear Factor Erythroid 2-Related Factor 2

NRG – Neuregulins

O:

O_2^- - Superoxide Anion

$OH^{\cdot}$ - Hydroxyl Radical

P:

PBS – Phosphate Buffered Saline

PDGF – Platelet Derived Growth Factor

PEP - Phosphoenolpyruvate

PI3K - Phosphoinositide 3-Kinase

PIK3CA - Phosphatidylinositol-4,5-bisphosphate 3-kinase, Catalytic Subunit Alpha

PIP_2/PIP_3 - Phosphatidylinositol 4,5 Bisphosphate/ Phosphatidylinositol 3,4,5 Triphosphate

PKM2 - Pyruvate Kinase M2

PRDX – Peroxiredoxin

PTEN - Phosphatase and Tensin Homologue

PTP – Protein Tyrosine Phosphatase

R:

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

RTK – Receptor Tyrosine Kinase

S:

S100A10 - S100 Calcium Binding Protein A10

SDS-PAGE – Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

Ser - Serine

SHP2 - Src-Homology 2 Domain-containing Phosphatase 2

shRNA – Short Hairpin RNA

SO^- - Sulfenic Acid

SO_2^- - Sulfinic Acid

SO_3^- - Sulfonic Acid

SOD – Superoxide Dismutase

Src - Proto-Oncogene Tyrosine-Protein Kinase Src

SRX – Sulfiredoxin

STAT - Signal Transducer and Activator of Transcription

T:

TBS-T – Tris Buffered Saline – Tween 20

TEMED - Tetramethylethylenediamine

TGF- α - Transforming Growth Factor α

TNF-α - Tumor Necrosis Factor–α

tPA - Tissue Plasminogen Activator

TR – Thioredoxin Reductase

TRX – Thioredoxin

V:

Val – Valine

VEGF – Vascular Endothelial Growth Factor

W:

WHO - World Health Organization

1. INTRODUCTION

1.1. Cancer

As defined by the World Health Organization (WHO), cancer is the uncontrolled growth and invasive spread of cells which can ubiquitously affect the body. Several decades of cancer research have led to the conclusion that cancer is not a single disease since it can occur almost anywhere in the human body. Cancer types are grouped into the following categories based on their cell of origin:

- **Carcinomas** - Cancers that originate from epithelial cells such as the cells that constitute the skin or tissues lining internal organs. Carcinomas constitute more than 80% of all existing cancers. Carcinomas constitute more than 80 % of all existing cancers. The subtypes of carcinoma include adenocarcinoma, basal cell carcinoma, squamous cell carcinoma and transitional cell carcinoma.

  Adenocarcinomas originate from epithelial cells that produce fluids or mucus (glandular tissues). Most cancers of the breast, colon, and prostate fall within this category.

  Basal cell carcinomas originate from the lower or basal layer (*stratum basale*) of the epidermis, that is, the outer layer of the skin.

  Squamous cell carcinomas (epidermoid carcinomas) originate from squamous cells, which are the epithelial cells that lie just beneath the outer surface of the skin. Squamous cells also form the lining of many other organs, including the intestines, lungs, stomach, bladder, and kidneys and could be the cells of origin for some cancers occurring in these organs.

  Transitional cell carcinomas originate from the transitional epithelium, or urothelium (multiple layers of epithelial cells which can expand and/or contract). This tissue is present in the linings of the ureters, bladder, renal pelvis, and a few other organs. Some cancers of the bladder, ureters, and kidneys fall within this category.
- **Sarcomas** - Cancers that originate from mesenchymal cells, including bone, cartilage, muscle, fat, blood vessels, or other supportive or connective tissues. Osteosarcoma is the most common bone cancer, and the most common types of soft

tissue sarcomas include Kaposi's sarcoma, leiomyosarcoma, malignant fibrous histiocytoma, liposarcoma, and dermatofibrosarcoma protuberans.

- **Leukaemia** - Cancers that usually originate from the bone marrow and lead to the production of large numbers of circulating abnormal blood cells. These cancers do not form solid tumours and are mainly constituted of circulating leucocyte progenitor cells, usually referred to as leukaemia cells and leukemic blast cells. Leukaemias are grouped based on how quickly the disease unfolds (acute or chronic) and on the type of blood cell of origin (lymphoblastic or myeloid). The four most common types include acute myeloid leukaemia, acute lymphoblastic leukaemia, chronic myeloid leukaemia and chronic lymphocytic leukaemia.
- **Lymphomas** - blood cell cancers that develop from lymphatic cells (T or B lymphocytes). Here, abnormal lymphocyte populations accumulate in lymph nodes, lymph vessels, as well as in other organs of the body where they form solid tumours (as opposed to leukaemias). The two main types of lymphomas include:

  Hodgkin's lymphoma – which originates from abnormal lymphocytes called Reed-Sternberg cells which originate from B cells.

  Non-Hodgkin's lymphoma – which originates from lymphocytes, can grow quickly or slowly and can arise from B or T lymphocytes.
- **Multiple Myeloma (plasma cell myeloma and Kahler disease)** - Cancer that originates from plasma cells, leading to the formation of abnormal plasma cells called myeloma cells which build up in the bone marrow and form tumors in the bones all throughout the body.
- **Melanoma** - Cancer that originates from the pigment-producing melanocytes in the basal layer of the epidermis. Most melanomas form on the skin, but can also form in other pigmented tissues, such as the eye (intraocular melanoma).
- **Neuroectodermic cancers** - cancers that originate from cells of the central and peripheral nervous system. They are named based on the type of cell of origin and the site of formation of the primary tumour in the central nervous system and include: gliomas, glioblastomas, neuroblastomas, medulloblastomas, astrocytomas and retinoblastomas.

Normal homeostatic functioning of the human body relies on tightly regulated communication and cooperation among cells and tissues. Defects in the aforementioned has several consequences, one of which being carcinogenic cellular transformation. Cancer results from the concerted mutations of a set of genes that normally control cell growth, proliferation, differentiation, DNA repair and apoptosis which are normal innate metabolic processes. These mutations give rise to oncogenes with dominant gain of function and tumour suppressor genes with recessive loss of function [1]. However, the conversion of normal human cells into malignant tumour cells is a multistep process occurring through a sequence of spontaneous and/or biochemically induced genetic alterations, each leading to different types of growth and survival advantages [2]. Cancer therefore turns out to be an unfortunate consequence of the multicellular mode of life. The physiological manifestations of carcinogenic mutations occur through six major alterations which include: independence from growth signals, circumvention of growth-inhibitory signals, bypass of apoptosis, unlimited replicative ability, induced and sustained angiogenesis, and invasion of tissues and metastasis [2]. Other recently identified cancer specific alterations include: significant reprogramming of energy metabolism in a bid to maintain continuous cellular proliferation and growth, particularly under adverse conditions such as hypoxia (low oxygen availability) and cancer cell evasion from immune cell mediated attack and elimination [3]. These alterations are enabled by two main characteristics, genome instability of cancer cells, which leads to random mutations that can enhance tumorigenic capabilities and a second enabling feature involves the inflammatory state of malignant and premalignant lesions that are orchestrated by cells of the immune system, promoting tumour progression [3]. The multiplicity of innate anticancer defence mechanisms bypassed by cancer cells demonstrate the multistep progressive onset of cancer and indicate the complexity of the acquired traits. It is therefore clear that there are significant differences between normal and cancer cell Signalling pathways which present significant bottlenecks in current cancer research and anticancer strategies. Identification and understanding of these differences keeps us in line as we assemble the bits of the complex cancer puzzle.

1.1.1. Epidemiology

Evidence of tumors found in fossilized dinosaur bones and the discovery of a 2700 year old human skeleton with clear signs of prostate cancer demonstrate that cancer has been around for a very long time, even though it is still a major health issue worldwide [4]. Within the past century, cancer incidence and prevalence in various populations has dramatically increased and seems to be on the rise. These increases are most probably a result of a generalized increase in life expectancy and greater exposure to cancer-causing substances from various sources in the environment - such as: pollution from industrialization, tobacco use, fuel combustion emissions from automobile engines and exposure to ionizing radiation (X-rays) etc. [4,5]. In 2012, the global number of new cases of cancer increased to about 14 million and it is expected to rise to an annual 19.3 million by 2025. The world's most prevalent cancer is lung cancer, constituting 13% of the total number of cases in 2012, followed by breast cancer (11.9%), colorectal cancer (9.7%), and prostate cancer (7.9%). The most common cancer among men is lung cancer (16.7%), followed by prostate (15%) and colorectal (10%) cancers. Among women breast cancer is the most common (25.5%), followed by colorectal (9.2%) and lung (8.7%) cancers [6,7]. The highest number of cancer cases occur in the less developed areas of the world, with 60% of cancers and 70% of cancer related deaths occurring in Asia, Africa, and Central and South America [6]. The greater prevalence in these regions can be linked to their large populations coupled to a lack of early detection and access to treatment which worsens the situation.

Country specific data for Portugal are similar to the global picture. The most common and lethal cancers in Portuguese males are lung (19.3%), colorectal (15.3%), prostate (11.8%), stomach (9.7%), and pancreatic (4.8%) cancers. In Portuguese females, breast cancer is most prevalent (16.9%), followed by colorectal (16.4%), stomach (9.4%), lung (8.0%) cancers and lymphomas and multiple myeloma (6.2%) [8].

Despite the marked exciting advances in cancer research, the global and individual country statistics indicate that cancer is still a major health problem nowadays and research efforts need to increase in order to meet up with the ever increasing challenges it presents.

1.2. Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are oxygen-containing chemical species that are produced as a result of incomplete reduction of oxygen (O_2) and are highly reactive towards DNA, proteins and lipids. Biologically active ROS include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical ($OH\cdot$) which possess intrinsic chemical properties that make them reactive to different biological targets. H_2O_2 is the most abundant ROS in cells and has special characteristics that makes it a relevant second messenger in signal transduction pathways. Unlike O_2^- (which cannot readily travel across membranes by virtue of its negative charge), H_2O_2 can freely diffuse through membranes, making it an ideal intracellular signalling molecule – the main modulator of redox signalling [9]. ROS are by-products of protein folding, cellular respiration (oxidative phosphorylation at the mitochondrial membrane) and end products of some metabolic reactions such as the oxidation of long chain fatty acids in the peroxisome and reactions catalyzed by some enzymes such as such as xanthine oxidases, nitric oxide synthases, cyclooxygenases, cytochrome P450 enzymes, NADPH oxidases and lipoxygenases [10]. Since ROS are much more reactive than O_2, they were initially labeled as harmful mediators of cellular damage through protein oxidation, lipid peroxidation and DNA damage. However, recent research has demonstrated important roles for ROS as second messengers in cell signalling, being implicated in biologically important cellular processes such as growth factor signalling, hypoxic signal transduction, autophagy, immune responses, proliferation, differentiation, migration and apoptosis [10,11]. This double-edge function of ROS appears contradictory considering the presence of highly robust and abundant cellular antioxidant systems including enzymes such as catalase, glutathione peroxidases, superoxide dismutases and thioredoxin peroxidases and systems that recycle the ROS scavenging proteins including the thioredoxin (TRX) and the glutathione (GSH) systems. However, it has been suggested that the concentration of ROS (H_2O_2) required for effective signalling is usually in the nanomolar range [11,12], whereas higher levels are associated with irreversible protein, lipid and DNA damage which could ultimately favor cancer initiation and progression (due to DNA mutagenesis) as well as other pathologies or cell death. Cellular ROS concentrations are therefore very tightly regulated and defective regulation could have devastating consequences.

1.2.1. Intracellular ROS production, regulation and signal transduction mechanisms

ROS are produced as a result of incomplete reduction of oxygen as by-products of protein folding, cellular respiration (mitochondrial oxidative phosphorylation) and end products of metabolic reactions (figure 1a). By virtue of its relative stability, spontaneous membrane diffusibility, and selective reactivity towards critical cysteine (Cys) residues, H_2O_2 has been identified as the main mediator of ROS-dependent signal transduction [13]. Most Cys residues in proteins have a pKa higher than 8.0 and will predominantly exist in the protonated thiol form (SH) at physiological pH (7.2 – 7.4). On the contrary, thanks to their local amino acid microenvironment, critical (reactive) Cys residues have a lower pKa (lower than physiological pH) and therefore predominantly exist as thiolate anions (S^-) at physiological pH, making them extremely more reactive than the SH form and specifically and selectively susceptible to H_2O_2 induced oxidation [14]. The universally accepted ROS dependent signalling mechanism occurs through the H_2O_2-mediated oxidation of reactive Cys residues within redox-sensitive proteins which triggers or blocks various signalling cascades[15]. The selective oxidation of reactive Cys residues (which are only present in a very restricted subset of proteins referred to as redox-sensitive proteins) and strict regulation and compartmentalization of H_2O_2 levels accounts for the specificity of redox signalling [11]. Optimal intracellular H_2O_2 levels (nanomolar range) provide an ideal environment for cellular signalling which maintains homeostasis in essential metabolic processes such as proliferation, differentiation and adaptation to metabolic and induced stress. However, supra-optimal H_2O_2 concentrations trigger oxidative damage and abnormal cell signalling causing pathologies like cancer, among others, or even cell death. Sub-optimal H_2O_2 levels on the other hand lead to cell signalling disruptions which result in loss of homeostasis[9] (figure 1b).

1.2.1.1. Intracellular ROS production:

Superoxide anion (O_2^-) can be produced as a result of the one-electron reduction of O_2 by NADPH oxidases (NOXs) in the cytosol and in the mitochondrial electron transport chain (ETC) complexes I, II, and III [16,17]. Mitochondria are thought to provide the lion's share of ROS in most cell types [10]. Once produced, O_2^- is immediately converted onto H_2O_2 by superoxide dismutase 1 (SOD1) in the cytosol or extracellular matrix. Extracellularly

produced H_2O_2 can diffuse through the cell membrane to the cytosol, where it performs its functions. In addition, O_2^- generated by the ETC (complexes I and II) in the mitochondria is released into the mitochondrial matrix where it is rapidly converted onto H_2O_2 by superoxide dismutase 2 (SOD2) [18]. ETC complex III-generated O_2^- is also released into the mitochondrial intermembrane space where it can cross through the voltage-dependent anion channels into the cytosol and where it is converted into H_2O_2 by SOD1 [19]. Direct production of H_2O_2 in the mitochondria by $p66^{Shc}$-mediated direct two-electron reduction of molecular oxygen to H_2O_2 has also been reported[20].

The very reactive hydroxyl radical (OH·), is produced as a result of the reaction between H_2O_2 and metal cations (Cu^+ or $Fe2^+$) through the Fenton reaction. Considering its very short half-life, lipid insolubility and strong oxidizing potential, the hydroxyl radical can cause irreversible oxidative damage to nearly any cellular macromolecule close to the area of its production. Since unrestrained levels of H_2O_2 lead to OH· formation, tight regulation of ROS (H_2O_2 in the nanomolar range) is critical for their role in redox-dependent signalling [13] **(**Figure 1b).

Enzymes such as cyclooxygenases, xanthine oxidase, nitric oxide synthase, lipoxygenases and cytochrome P450 enzymes are also known to produce ROS to a lesser extent and their overall contribution depends on the cell type. Furthermore, the peroxisomes and endoplasmic reticulum have also been described as minor sources of ROS [10](Figure 1.1a). H_2O_2 is produced as an end product in several oxidation pathways in the peroxisomes such as in the β-oxidation of long-chain fatty acids, by a wide range of enzymes including cytochrome P450 and as a by-product of protein oxidation in the endoplasmic reticulum (ER) [21]**.** However, it is noteworthy that the specific targets of ROS are usually proximal in location to the ROS generating systems and their production is highly compartmentalized within specific cellular locations.

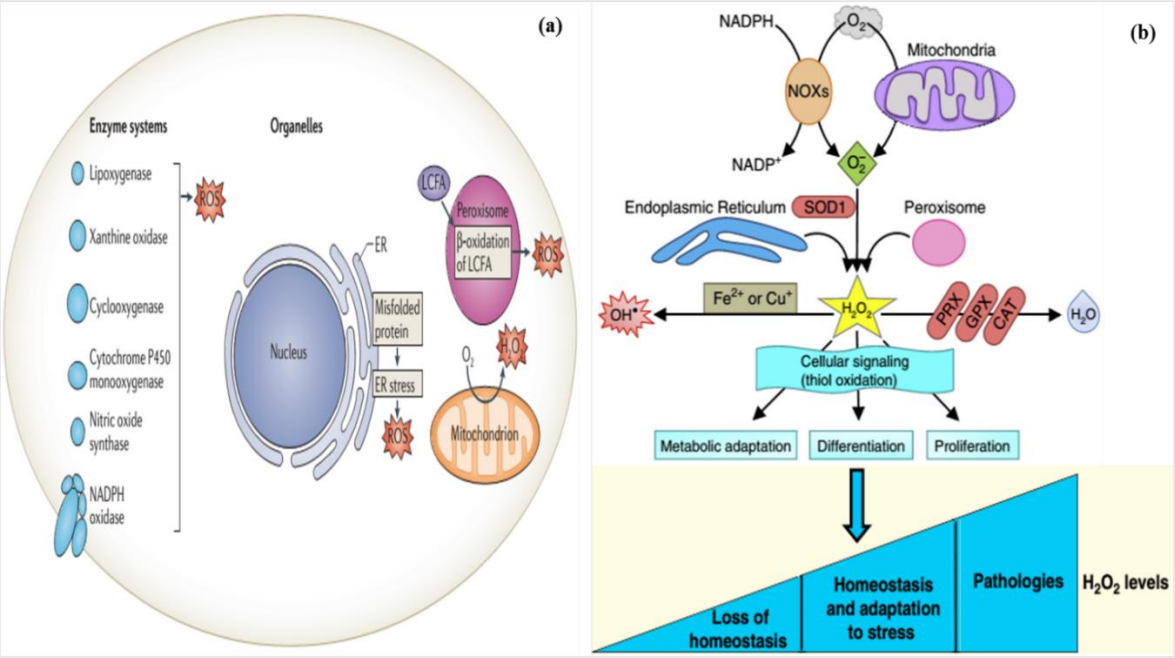

Figure 1.1: Intracellular sources (a) and regulation (b) of ROS. ***(a)*** *Various organelles such as the mitochondria, the endoplasmic reticulum and peroxisomes as well as various enzymes, including oxidases and oxygenases, generate ROS as part of their enzymatic reaction cycles.* ***(b)*** *The produced O_2^- is rapidly converted into H_2O_2 by SOD1. H_2O_2 can either function as a Signalling molecule regulating several biological processes, including metabolic adaptation, differentiation, and proliferation, or be dismutated to H_2O by ROS scavenging enzymes peroxiredoxin (PRDX), glutathione peroxidase (GPX), and catalase (CAT). H_2O_2 can also react with metal cations (Fe2+ or Cu+) to produce the hydroxyl radical ($OH\cdot$), which causes irreversible oxidative damage to lipids, proteins, and DNA. Suboptimal H_2O_2 levels lead to a disruption of cell Signalling causing a loss of homeostasis, while supra-optimal H_2O_2 levels lead to oxidative damage and aberrant cell Signalling resulting in pathologies such as cancer* [9,10,13].

1.2.1.2 Intracellular regulation of ROS levels

In order to curtail the accumulation of H_2O_2 and the acute toxicity of $\cdot OH$**,** cells possess powerful antioxidant systems which temporally and spatially regulate intracellular ROS concentrations. The rapid SOD-mediated dismutation of O_2^- to H_2O_2 prevents the accumulation of the highly reactive and damaging ROS, O_2^- [22], even though it leads to the production of another more stable ROS (H_2O_2). The enzymatic conversion of H_2O_2 to water (H_2O) and O_2 by antioxidants including glutathione peroxidases (GPXs), thioredoxin peroxidases (e.g. peroxiredoxins (PRDXs), annexin A2 and catalase), coupled with the GSH and TRX systems are together responsible for keeping H_2O_2 levels in check (Figure 1.1b). While catalase is only found in the peroxisomes of most cells, glutathione peroxidases

(GPXs), peroxiredoxins (PRXDs) and annexin A2 can be found in various cell compartments including the cytosol, mitochondria, nucleus and ER.

Peroxiredoxins (PRDXs) are thioredoxin peroxidases that are divided into three classes: typical 2-cysteine peroxiredoxins (PRDX I-IV), atypical 2-cysteine peroxiredoxins (PRDX V), and 1-cysteine peroxiredoxins (PRDX VI). They are a highly abundant group of ROS scavengers which contain a conserved reactive Cys (referred to as the peroxidatic Cys (Cp) [Cys47] to indicate its sensitivity to peroxide oxidation) in their active site which is oxidized in the presence of H_2O_2 (forming a -Cys-SO^- intermediate or intra and inter-molecular disulfide bonds between the catalytic cysteine and the resolving cysteine) and subsequently reduced by the TRX system in every complete catalytic cycle. NADPH is then used as an electron donor for the thioredoxin reductase (TR) catalyzed reduction of the oxidized TRXs [23]. PRDXs are also special in their ability to undergo a reversible transient hyperoxidation mediated by a second H_2O_2 molecule, forming sulfinic acid (-Cys-SO_2^-) which temporally inactivates the protein and favors ROS accumulation during signalling events, as will be elaborated later [24]. Sulfiredoxin (SRX) reduces the hyperoxidized PRDX back to the -Cys-SO^- intermediate through an ATP- dependent reaction, making it available for further redox cycles [25]. By virtue of their abundance, PRDXs are ideal candidates for scavenging of H_2O_2.

Glutathione peroxidases (GPX1-8) catalyze the reduction of H_2O_2 by oxidizing the reduced form of glutathione (GSH) forming a glutathione disulfide (GSSG) which is reduced back to GSH by GSH reductase using NADPH as an electron donor [13]. They have higher reaction rate constants but are less abundant than the PRDXs and are thought to be more implicated in H_2O_2 scavenging during oxidative stress (intracellular supraoptimal elevation of H_2O_2 levels) [26].

NADPH plays a critical rate limiting role in the production (through NOXs) and scavenging (electron donor in PRDXs and GPXs reactions) of ROS. In spite of the high abundance of ROS scavengers, some H_2O_2 (nanomolar concentrations) manages to bypass these robust antioxidant systems to participate in signalling pathways.

1.2.1.3 Intracellular ROS (H_2O_2) mediated Signalling mechanisms

By virtue of its selective specificity towards reactive Cys residues, spontaneous membrane diffusibility and stability, H_2O_2 is considered the main ROS involved in redox signalling. H_2O_2 carries out its signalling function by oxidizing reactive Cys residues within redox-sensitive proteins [15]. H_2O_2 molecules oxidize the thiolate anions to the sulfenic intermediate (SO^-) which changes the conformation and activity of redox-sensitive proteins and thereby impacts on intracellular signalling. Supraoptimal H_2O_2 levels could lead to hyperoxidation of the SO^- to form sulfinic (SO_2^-) and sulfonic (SO_3^-) acids. Hyperoxidation to SO_3^- is irreversible and can be prevented by the rapid conversion of SO^- into sulfenic-amide (S–N), disulfide (S–S) bonds, or glutathionylation. SO_2^- can be reduced back to the SO^- intermediate by sulfiredoxin (SRX). The resulting oxidized proteins are restored to their reactive reduced state through the actions of TRXs and GRXs (figure 1.2a). Another well described, but less common post-translational oxidative modification of reactive Cys is nitrosylation (RSNO) [27].

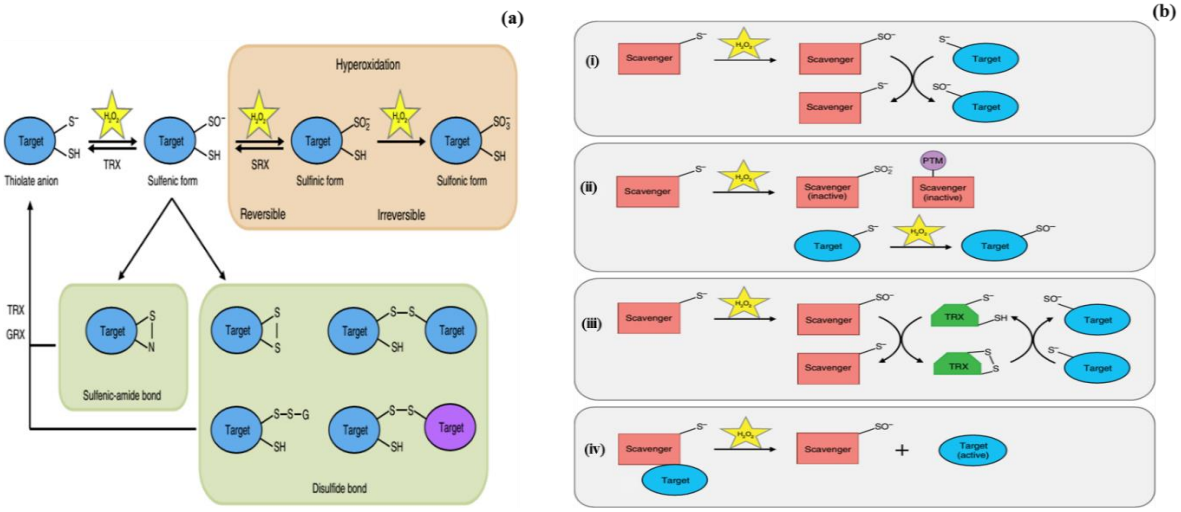

Figure 1.2: H_2O_2-mediatedoxidation of reactive Cys (a) and Signalling mechanisms [13]. ***(a)*** *H_2O_2 readily oxidizes the thiolate anions (S-) of reactive Cys yielding sulfenic acid (SO^-) which modifies protein activity. High levels of H_2O_2 can lead to hyperoxidation to generate sulfinic (SO_2^-) and sulfonic (SO_3^-) acids (brown box). Oxidation to SO^- and SO_2^- is reversed by TRX and SRX respectively. Formation of SO_3^- is irreversible and is prevented by the formation of reversible disulfide (S–S) or sulfenic-amide (S–N) bond (green box.* ***(b)*** *(i) The redox relay mechanism - ROS-scavenging proteins are initially oxidized by H_2O_2 and subsequently transfer the oxidation to the target protein. (ii) Floodgate mechanism - transient reversible inactivation of ROS scavenging proteins, allowing for localized accumulation of H_2O_2 for Signalling (iii) Redox relay mechanism with scavenging enzyme (TRX) as intermediate. (iv) Oxidative release mechanism – activation of target protein by dissociation from oxidized scavenging enzyme.*

Recent studies have identified several mechanisms of H_2O_2-mediated oxidation of reactive Cys residues and signalling regulation, including: redox relay, floodgate, oxidative release and other likely mechanisms as described below.

(i) The redox relay mechanism:

In this mechanism, the ROS-scavenging proteins are initially oxidized by H_2O_2 and subsequently transfer the oxidation to target protein(s) causing activating or deactivating conformational changes (figure 1.2b (i)). For example, in response to H_2O_2, the apoptosis signal regulating kinase 1 (ASK1) protein is oxidized, leading to the phosphorylation/activation of the pro-apoptotic MAPKs, p38 and JNK. This occurs through the formation of a PRDX1–ASK1 mixed disulfide intermediate [28]. Another redox relay type of mechanism has been proposed in which there is an intermediate step involving oxidation of the antioxidant proteins, PRDX, GSH or TRX prior to the transfer of the oxidation to the target protein or vice versa (figure 1.2b (iii)). An example of this type of redox relay was described in a study with 5'-AMP-activated protein kinase (AMPK), a redox sensitive kinase, where it was observed that, during energy starvation, AMPK molecules become oxidized and aggregate (by forming intermolecular disulfide bonds at Cys130 and Cys174 residues), which prevents its phosphorylation/activation by AMPK kinases. Here, TRX1 and/or PRDX supports AMPK activation by reducing these key residues, thereby permitting its activation for the regulation of metabolism and survival during energy stress [29].

(ii) **The flood gate mechanism**:

Another mechanism of H_2O_2 dependent signalling occurs through a transient reversible inactivation of ROS scavenging proteins, allowing for localized accumulation of H_2O_2 for signalling events [30,31] (figure 2b (ii)). It is proposed as a 'flood gate' model in the sense that the temporal inactivation of some key enzymes (e.g. PRDXs) causes local flooding of H_2O_2 for signalling, while the other scavengers remain active to keep ROS levels in check and prevent non-localized diffusion of H_2O_2. The inactivation of these scavenging enzymes has been proposed to occur through temporal hyperoxidation [32] and/or phosphorylation [24] causing reversible deactivating conformational alterations. The unique ability of PRDXs to be

reversibly hyperoxidized to sulfinic acid (-Cys-SO_2^-) coupled with the intracellular presence of the sulfinic scavenging enzyme (SRX) demonstrates the possibility of occurrence of this mechanism *in vivo*. Also, the *in vivo* existence of this model has been described in Zebra fish models[33].

(iii) The oxidative release mechanism:

An additional proposed mechanism of H_2O_2-mediated signalling is the initiation of signalling by the activation of a target protein after its release from a complex with a redox regulatory enzyme. In this case, the antioxidant protein inactivates the target protein by direct binding. In the presence of H_2O_2 the antioxidant protein suffers an oxidative conformational change which severs the interaction, thereby activating the released target protein. This model has been described for the interaction between ASK1 (a pro-apoptotic protein) and TRX, whereby ROS-mediated separation of the aforementioned interaction releases ASK1 that can oligomerize and activate signalling pathways [34] .

In line with the mechanisms described above, it is evident that in addition to their modulatory functions, the ROS scavenging enzymes may also be active sensors and transducers of redox signals, thereby participating actively in signalling events. The identification of the role of ROS in several metabolic signalling pathways coupled with the discovery of new redox-sensitive proteins and elucidation of their signalling pathways is providing useful knowledge in normal and cancer cell signalling with useful applications in the development of anticancer networks. Further research into these signalling mechanisms and pathways will uncover more mysteries regarding the initiation, progression, treatment and prognosis of cancer.

1.2.2. Physiological and metabolic outcomes of ROS-dependent Signalling

One of the first demonstrations of a role for ROS as second messengers in cell signalling was the observation of a transient surge in ROS generation which precedes increases in tyrosine (Tyr) phosphorylation occurring after epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin, interleukin-1 (IL-1) and tumor necrosis factor–α (TNFα) stimulation [35,36]. Since then, essential links have been established between growth factors, the production of ROS and Tyr phosphorylation, whereby the binding of growth factors to

their receptors leads to the activation of NOX which produces H_2O_2 for downstream signalling. [37]. The detailed mechanism for growth factor induced generation of H_2O_2 and physiological effects are discussed in detail later in this work. Growth factor induced production of ROS affect downstream signalling pathways by ROS-mediated oxidation of reactive Cys leading to Tyr kinases and PTPs activation and inactivation, respectively. The oxidation of reactive Cys residues is also important as a means of regulation of the recently described large family of Cys-dependent proteases. For instance, H_2O_2-mediated inactivation of ATG4 (an essential autophagy protein) has been observed, which leads to augmented autophagosome formation [38].

H_2O_2 is a second messenger in several signalling pathways regulating a variety of cellular processes, including proliferation, differentiation, metabolism, survival and apoptosis. Some examples of such pathways include the mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinase (PI3K) cascade which are activated in response to ROS resulting from growth factor stimulation and/or oxidative stress [39]. Within the MAPK pathway, ASK1, an upstream MAPK kinase kinase, is activated through H_2O_2-mediated oxidation of TRX, leading to its dissociation from ASK1 which in turn becomes activated, resulting in downstream activation of the JNK and p38 MAPK signalling pathways leading to apoptosis in response to oxidative stress [40,41]. The PI3K pathway plays a key ROS-dependent role in cell proliferation and survival in response to growth factors, hormones, and cytokines stimulation. PI3K catalyzes the synthesis of phosphatidylinositol 3,4,5 triphosphate (PIP_3) from phosphatidylinositol 4,5 bisphosphate (PIP_2), leading to the recruitment of protein kinase B (AKT) and further downstream signalling events that mediate proliferation and survival. Phosphatase and tensin homologue (PTEN) is a major negative regulator of this pathway by dephosphorylating PIP_3 back to PIP_2 [42,43]. Here, through PTEN, the PI3K pathway is subjected to reversible redox regulation whereby H_2O_2 oxidizes and inactivates PTEN (via disulfide bond formation between the catalytic domain Cys-124 and Cys-71 residues) leading to the activation of the PI3K pathway [44,45].

The identification of redox-regulated transcription factors has led to the elucidation of ROS-mediated transcriptional regulation. One of the first examples of this type of mechanism was the description of the redox regulated DNA binding of the Fos-Jun heterodimeric

transcription factor complex. These studies showed that binding of Fos-Jun to the activator protein-1 (AP-1) DNA sequence motif was mediated by a conserved reactive cysteine residue located in the DNA-binding domains of Fos and Jun [46–48]. This heterodimeric complex formed by Fos and Jun binds to transcriptional control elements containing AP- 1 binding sites and acts as an intermediary transcriptional regulator in signal transduction processes. Oxidation with diazenedicarboxylic acid *bis* [N,N-dimethylamide] was shown to inhibit the binding of Jun monomer and Fos-Jun heterodimer to DNA, an effect which was reversed by incubation with reducing agents, thereby confirming their redox regulation [47]. Another example involves the forkhead box protein O (FOXO) family of transcription factors, whereby increased ROS levels favors redox-dependent interaction (formation of protein–protein disulphide bridges) between FOXO4 and the nuclear import receptor transportin 1 leading to nuclear import of FOXO4 and consequent activation of transcription of target genes mediated by this protein [49]. Another example was presented in a study which described the redox-dependent activation of the nuclear factor erythroid 2-related factor 2 (Nrf2), which plays a major role in the protection of cells from xenobiotic and oxidative damage [50]. Under normal physiological conditions, Nrf2 is rapidly degraded via the ubiquitin–proteasome pathway in a Kelch-like ECH-associated protein 1 (Keap1)-dependent manner whereby, Nrf2 is continuously ubiquitinated by the Cullin 3 (Cul3)–Keap1 ubiquitin E3 ligase complex leading to its proteosomal degradation [51]. During oxidative stress, the reactive cysteine residues of Keap1 are oxidized, leading to stabilization of Nrf2 and its translocation and accumulation in the nucleus where it binds to antioxidant response elements (ARE) within the antioxidant genes promoters, thereby enhancing the overall cellular ROS scavenging capacity [50]. Redox regulation of other transcription factors including NF-κB, STAT3, HIF-1 and p53 has been also been described [52–56]. The aforementioned transcription factors all possess reactive cysteine residue(s) within their DNA binding domains that can be readily oxidized by cellular ROS leading to loss of binding to their promoters/DNA.

The enhancement of cellular ROS production by growth factor/growth factor receptor (GF/GFR) induced NOX activation has been adequately linked to increased rates of mitosis, cell transformation and tumorigenicity as demonstrated in NOX1 expressing NIH-3T3 fibroblasts where coexpression of catalase and other antioxidant treatment reversed this

effect [57]. Another study using NIH 3T3 cells transformed with H-Ras showed that these cells produced large quantities of ROS compared to control cells, which induced cell cycle progression and consequent proliferation in the absence of growth factors. This was reverted by treatment with the antioxidant *N*-acetyl-L-cysteine (NAC) [58]. Supraoptimal concentrations of H_2O_2 can induce the upregulation of Fas expression (a death signal), increase intracellular ion uptake, and induce mitochondrial DNA damage , all of which can trigger apoptosis [59].

ROS are therefore deeply and intricately involved in various signalling pathways as potent second messengers with various physiological outcomes in normal living cells. However, by virtue of the non-specific protein, lipid, and DNA damage caused by high levels of ROS, we can already predict their involvement in tumor initiation and progression (due to DNA mutagenesis and activation of proliferative and pro-survival signalling pathways).

1.2.2.1 The role of ROS in cancer

ROS play a complex role in cancer and have been implicated in the initiation and progression of several types of cancer. The uncontrolled proliferation and high metabolic rate of cancer cells leads to the production of high levels of ROS which keeps them in an almost permanent state of oxidative stress [60]. In order to counteract the possible ROS-induced DNA, protein and lipid damage, cancer cells are known to up-regulate the expression of cellular antioxidant proteins. ROS-induced DNA damage, can lead to genomic instability which usually induces further increments in intracellular ROS levels, thereby effecting cellular senescence and apoptosis which can prevent genetically damaged cells from further proliferation and hence impede tumor formation. However, the up-regulation of antioxidants can on one hand be beneficial by reducing ROS levels and by doing so reducing DNA damage, while, on the other hand, they could help cancer cells to adequately bypass senescence or apoptosis, and consequently speed up tumor formation [10]. ROS and antioxidant levels are therefore intricately connected to cancer cell metabolism and seem to have a double-edged sword function.

ROS scavenging depends mainly on the availability of reducing equivalents provided by NADPH which is produced via the pentose phosphate pathway. The increased need for these

reducing equivalents in cancer cells pushes them to divert glucose metabolism towards the anabolic pentose phosphate pathway [61]. Pyruvate kinase is a glycolytic enzyme that converts phosphoenolpyruvate (PEP) to ATP and pyruvate during normal homeostasis [61]. Recently, it was reported that cancer cells express pyruvate kinase M2 ([PKM2] a specific redox-sensitive isoform), and increased ROS levels target a reactive Cys residue in PKM2 which when oxidized leads to the inhibition of PKM2 and consequent diversion of glucose away from the normal catabolic pyruvate production towards the anabolic pentose phosphate pathway. This leads to increased NADPH production for redox buffering by GSH and other antioxidant systems, to compensate for the aberrant oxidative state [62].

Another important link between cancer cell metabolism and ROS comes from recent studies of autophagy (cellular self-cannibalism). There is increasing evidence showing that part of the metabolic reprogramming in cancer cells involves upregulation of autophagy [10]. Autophagy is mainly induced by starvation, which in turn leads to increments in intracellular ROS levels, which induce autophagy-mediated recycling of macromolecules and organelles to fuel mitochondrial metabolism in a bid to provide the much needed energy in the perpetually hungry cancer cells [63]. This phenomenon was observed to be more common in Ras-expressing tumors whereby Ras triggers autophagy by ROS-induced hypoxia inducible factor (HIF-1α)-mediated activation of BNIP3 [64]. This ROS-induced autophagy-mediated stress response mechanism seems to be particularly important in cancer cells to provide energy and metabolic substrates, to recycle toxic damaged proteins and organelles and to limit cell death and tissue inflammation, thereby favoring survival and tumor growth. The clearance of damaged mitochondria (mitophagy), which are potential sources of ROS (which trigger cell death through ROS-induced necrosis), also occurs during autophagy further contributing to survival [65]. ROS-induced autophagy addiction therefore stands out as a possible cancer cell survival mechanism as demonstrated by the energetically compromised state observed in autophagy-deficient cancer cells with tumorigenic mutations in Ras oncogenes under starvation [66]. Another important outcome of ROS-induced autophagy-mediated cancer cell survival under oxidative stress is the potential activation of autophagy as a chemotherapeutic and radiation resistance mechanism whereby ROS induce the assembly and maturation of the autophagosome which removes dysfunctional mitochondria

and proteins, as demonstrated in human hepatocytes [67]. There are therefore strong links between the cancer cells' autophagic flux, their intracellular redox state, growth, survival and resistance to chemotherapy and radiotherapy.

Gain of function mutations in the PI3K pathway leading to its constitutive activation (and simultaneous ROS mediated inactivation of PTPs and PTEN) is a main mechanism of malignant tumor development and this pathway stands out as one of the most mutated in several types of cancer, with mutations that lead to cancer cell PI3K addiction, and understandably so, due to its sustaining effect on proliferation and survival [68]. In this regard, the perpetual state of oxidative stress in cancer cells seems to be an advantage. The identification of a positive correlation between the RAS oncogene expression and increased ROS production with the observation of concomitant increases in proliferation in cancer cells transformed with Ras provided the first hints linking ROS with cancer and created avenues for further research [58]. Here, NIH 3T3 cells transformed with a perpetually active isoform of Ras (v-H-Ras or EJ-Ras) were observed to produce large amounts of O_2^- and had higher rates of proliferation compared to control cells, an effect which was reversed by treatment with the chemical antioxidant N-acetyl-L-cysteine [58].

ROS have also been implicated in metastasis, which stands out as a major piece of the cancer puzzle. Mitochondrial ROS (mROS) production has been associated with the metastatic ability of cancer cells, as demonstrated in a recent study in which endogenous mitochondrial DNA (mtDNA) in poorly metastatic mouse tumor cell lines was replaced with mtDNA from highly metastatic cell lines, and vice versa. These studies showed that recipient cancer cells acquired the metastatic potential of the transferred mtDNA in vivo [69]. The aforementioned acquired metastatic potential was confirmed to result from mtDNA mutations leading to a deficiency in respiratory complex I (NADH dehydrogenase) activity which favored the overproduction of ROS, leading to higher expression of the antiapoptotic *MCL-1* (myeloid cell leukemia–1), *HIF-1α* and *VEGF* neoangiogenic genes, creating the right environment for metastasis [69]. A significant suppression of this effect was observed with pretreatment with ROS scavengers [69]. These outcomes indicate the possible implication of mtDNA-mediated ROS production in metastatic tumor progression. In addition, as opposed to nuclear DNA, mtDNA is not protected by histones and is therefore more exposed to ROS, indicating a

higher rate of ROS-induced mtDNA mutagenesis compared to nuclear DNA which can be inherited by subsequent cellular generations, thereby favoring the evolution of the malignant phenotype [70,71]. ROS-induced mitochondrial genome instability has also been associated with increased growth and invasiveness in colon and prostate cancers [72,73]. This demonstrates an important role for ROS in cancer initiation through mtDNA mutagenesis. In another study with normal and prostate cancer cells, a significant increase in ROS generation was observed in cancer cells, with much higher levels in the more invasive PC3 cells indicating the critical role of ROS in promoting metastatic phenotypes which was confirmed by cyclin B–dependent G2-M cell cycle arrest upon treatment with diphenyliodonium (NOX inhibitor) which impeded proliferation by modulating the activity of growth signalling pathways including extracellular signal-regulated kinase (ERK1/2), p38 MAPK and AKT [72].

The ability of cancer cells to adapt to acute and chronic hypoxia is critical for their survival, especially in more advanced tumors which are subjected to a constant oxygen depleted state (hypoxia) [74]. In this respect, hypoxia-induced mtROS-mediated signalling leads to the stabilization of the HIF-1α transcription factor which stimulates survival-supporting gene expression. This effect was confirmed by the mitochondrial intermembrane space (IMS)-PRDX5 mediated inhibition of hypoxia-induced oxidant signalling and concurrent decrease in HIF-1α stabilization in pulmonary artery smooth muscle cancer cells overexpressing PRDX5 which led to decreased survival [74,75].

In summary, as cells acquire a cancerous phenotype, they trigger ROS-dependent metabolic cascades that favor incessant proliferation, survival and metastasis leading to further ROS generation and keeping the cancer cells at a perpetual near toxic oxidative state [76]. To curtail this, cancer cells often acquire a more glycolytic metabolism that increases NADPH production via the pentose phosphate pathway to ensure an adequate supply of reducing equivalents to support the cellular antioxidant systems in conjunction with the activation of redox-dependent transcription factors (e.g. Nrf2) which upregulate the expression of antioxidant proteins [62,77].

Though not exhaustive, the information in the above paragraphs shows that ROS have varied effects on normal and malignant tumor cells, and further research is necessary to fill in the

knowledge and mechanistic gaps in ROS-mediated signalling in order to determine the precise role of ROS in cancer and other diseases and identify new potent therapeutic targets.

1.3. Epidermal Growth Factor (EGF)-induced Signalling

Pioneering research on the epidermal growth factor receptor (EGFR) pathways brought to light the widely used concept of linear signalling initiated by ligand-bound receptors and propagated downstream via multiple effectors[78]. Since its discovery in the early 1970s, epidermal growth factor (EGF) has been adopted as one of the most biologically potent and best characterized growth factor (at the physical, chemical, and biological levels) [78,79]. EGF/EGFR signalling is one of the most capital pathways involved in the regulation of growth, proliferation, differentiation, and survival in normal and transformed cells and has been widely studied [80,81].

As described in the pioneering work by Cohen and Carpenter as well as subsequent crystallography studies, human EGF (one of the 11 known human EGF-family ligands) is a 53 amino acid single-chain polypeptide which contains six Cys residues that form three disulfide bonds and is encoded by a 4.8-kb mRNA transcript from a 110 kb long gene containing 24 exons and located on human chromosome 4q25 [79,82–84]. It is initially synthesized as a large (1207 amino acids) preproEGF molecule containing a hydrophobic signal peptide and an N-terminal transmembrane domain which has been shown to bind and stimulate EGFR on adjacent cells [85,86]. The biologically active EGF peptide is produced by proteolytic cleavage of the N-terminal domain of the preproEGF which releases it for its autocrine or paracrine ligand function [86,87]. EGF is expressed in several adult tissues and predominantly in the epithelial cells of the gastrointestinal tract [87]. EGFR is a 1210 amino acid transmembrane glycoprotein which is a member of the ErbB family of receptor tyrosine kinases (RTK) which is constituted by four distinct receptors: the EGFR (ErbB-1/human epidermal growth factor receptor 1 [HER1]), ErbB-2 (neu, HER2), ErbB-3 (HER3) and ErbB-4 (HER4), all having a less conserved extracellular ligand-binding domain, a single hydrophobic transmembrane domain and a highly conserved cytoplasmic tyrosine kinase-containing domain and are activated following binding of growth factors of the EGF-protein family which are usually released by autocrine or paracrine secretion [83,84,88,89]. The receptor

therefore has two main functions: ligand binding and an intrinsic protein Tyr kinase activity (which includes autophosphorylation of the receptor in response to EGF binding) [90]. Based on their ErbB-receptor binding, EGF-related peptide growth factors have been divided into three groups with the first group comprising EGF, amphiregulin, epigen and transforming growth factor α (TGF-α) which specifically bind to the EGFR; the second group includes heparin-binding growth factor (HB-EGF), epiregulin and betacellulin which are dually specific, binding both ErbB-4 and EGFR. The third group is composed by neuregulins, some of which bind only to ErbB-4 (NRG-3 and NRG-4) or to ErbB-3 and ErbB-4 (NRG-1 and NRG-2) [86,91–93]. The binding of EGF to the extracellular ligand-binding domain of EGFR prompts receptor dimerization, which normally brings the two cytoplasmic tyrosine kinase domains of the receptors in close proximity for autophosphorylation and consequent activation of the intrinsic tyrosine kinase activity [82]. These phosphorylated residues serve as anchoring sites for Src homology 2 (SH2)-containing proteins and phosphotyrosine binding (PTB) domains which, when recruited trigger several downstream intracellular signalling pathways such as the Ras/Raf/ MAPK (proliferation) and the PI3K/Akt pathways (proliferation and survival) [94]. Interestingly, ROS have also been implicated in apoptosis, mainly during oxidative stress. Endogenous H_2O_2, produced by the mitochondria and low density lipoprotein oxidation was observed to induce apoptosis via JNK activation in cells in conjunction with upstream Src-dependent activation of EGFR [95,96].

Considering the importance of the physiological effects of EGF-induced signalling in the maintenance of cancer and its hallmarks, it would be logical to expect its involvement in the initiation and progression of various cancer types [87]. Also, considering the various components of the pathways activated by the EGF/EGFR interaction, it is noticeable that these are dependent in a large part on ROS signalling, as described in previous sections of this thesis. It would therefore be expected that EGF binding would lead to signalling events that trigger the production of ROS as potent second messenger signal transducers which further activate other signalling pathways with various physiological outcomes.

1.3.1. EGF-induced ROS production

Several ligands have been identified that carry out their signalling functions by stimulating the production of H_2O_2. These include peptide growth factors such as EGF, PDGF, basic fibroblast growth factor (bFGF), granulocyte-macrophage colony- stimulating factor (GM-CSF) and insulin; heterotrimeric guanosine 5′-triphosphate (GTP)-binding protein (G protein)-coupled receptors (GPCRs) agonists such as thrombin, angiotensin II, thyrotropin, lysophosphatidic acid (LPA), parathyroid hormone, sphingosine 1-phosphate, endothelin, serotonin, acetylcholine, bradykinin and platelet-activating factor; and cytokines such as interferon-γ, transforming growth factor-β (TGF-β1), interleukins 1 and 3, TNF-α and the T cell antigen receptor [36]. However, the ubiquitous involvement of EGF/EGFR and its homologues in a wide range of malignancies [97–99] as ROS-mediated drivers of some of the main hallmarks of cancer makes it an appropriate candidate for our present study.

As described in the above paragraphs, the initial triggering events in response to EGF/EGFR interaction involve the activation of tyrosine kinase activity followed by several downstream events mediated by H_2O_2 [82]. This possibility led Bae *et al.* to investigate EGF-induced ROS generation and its role in tyrosine phosphorylation, which was one of the first studies in which EGF stimulation was adequately associated with ROS generation for signalling, especially in the ever active cancer cells [35]. In the aforesaid study, it was unambiguously ascertained that EGF induces ROS production as demonstrated in A431 human epidermoid carcinoma and NIH 3T3 cells whereby significant increases in 2',7'-dihydrodichlorofluorescein diacetate (DCFH-DA) fluorescence were observed after five minutes of EGF treatment. This result was confirmed to be primarily attributable to H_2O_2 on the basis of the disappearance of dichlorofluorescein (DCF) fluorescence after introduction of catalase (H_2O_2 scavenger) into the cells by electroporation. In addition, the tyrosine phosphorylation of EGFR and phospholipase C-ɣ1 after EGF-induction was completely blocked in A431 cells containing exogenous catalase indicating a reliance of the tyrosine phosphorylation of the aforementioned proteins on EGF-induced increases in H_2O_2 levels [35]. Also, in order to gain more insight into the role of the EGFR activity and/or autophosphorylation sites in EGF-induced ROS production, cell lines expressing either the wild-type EGFR, a truncated EGFR without the C-terminal 126 amino acids (CD-126 EGFR

[absence of four out of five autophosphorylation sites]) or a tyrosine kinase-negative EGFR were treated with EGF, after which significant increases in H_2O_2 levels were observed in the wild-type or CD- 126 EGFR but not in the cells with the catalytically inactive mutant suggesting that the receptor tyrosine kinase activity, and not necessarily autophosphorylation is needed for the EGF-induced ROS production. Furthermore, it was also hypothesized in this study that since all PTPs contain a reactive Cys moiety in their active site PTPs may be targets of EGF-induced intracellular H_2O_2 [35,100]. This hypothesis was confirmed in later studies in which the inhibition of PTEN (tumor suppressor) and PTPs was observed in various cell types and was confirmed to result from H_2O_2-mediated oxidation of their catalytic Cys residues in response to EGF and/or PDGF stimulation [44,101–104].

A precise mechanism for EGF-induced H_2O_2 generation and initiation of signalling events has been recently proposed by various studies, in which the stimulation of cells with EGF leads to the activation of PI3K, which phosphorylates PIP_2 forming PIP_3. PIP_3 then activates guanine nucleotide exchange factors such as Ras which activate the Rac proteins (exchange of Rac-bound GDP for GTP) leading to the assembly and concomitant activation of the NOX1 complex, resulting in the production of O_2^- that is then converted to H_2O_2 by SOD. The H_2O_2 so produced mediates the transient inactivation of neighboring cytoplasmic PRDX molecules by reversible hyperoxidation which in turn promotes local accumulation of H_2O_2. The accumulated H_2O_2 molecules oxidatively inactivate PTEN (by causing the formation of a disulfide bond between Cys124 and Cys71 in its active site) and PTPs (e.g. PTP1B and SHP2 by oxidation of the conserved reactive Cys present at the catalytic site). The inactivation of PTEN leads to further increases in the levels of PIP_3 which triggers downstream signalling pathways such as the PI3K-AKT and Ras-ERK pathways with physiological effects such as proliferation, differentiation, growth, migration, survival, angiogenesis and metastasis [32,36,44,103,105,106]. In addition, EGFR has also been reported to have a reactive Cys in its active site (Cys-797), which, when oxidized, enhances its Tyr kinase activity and subsequent signalling, indicating a positive feedback function of the EGF-induced H_2O_2 on EGFR [106]. The H_2O_2 signal is then quenched by the reactivation of PRDXs and the resultant scavenging of H_2O_2 in combination with the NADPH-dependent

reactivation of oxidized PTEN by TRX, restoring its 3-phosphatase function (dephosphorylation of PIP_3 to PIP_2) [32,36,44,103,105,106] (figure 1.3).

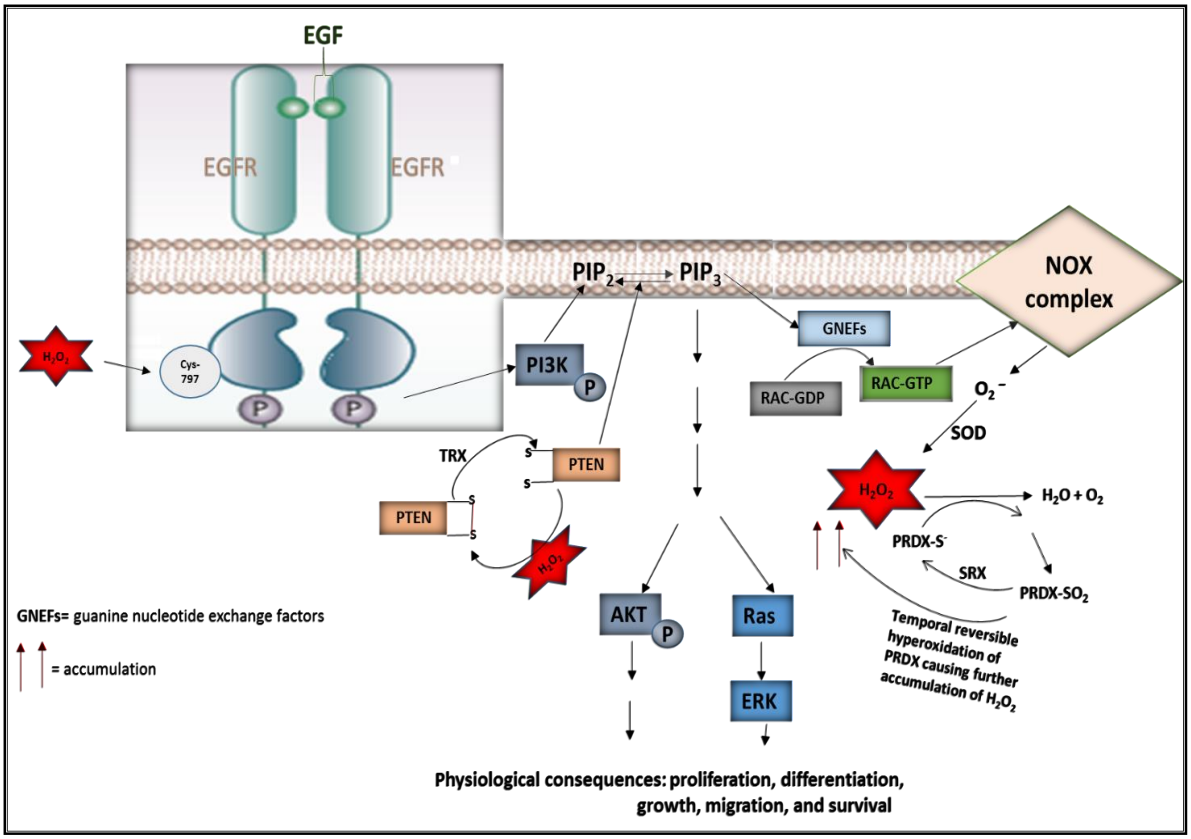

Figure 1.3: Mechanism for EGF-induced H_2O_2 generation and initiation of Signalling events[106]. *EGF binding to EGFR induces receptor autophosphorylation, leading to activation of PI3K, which phosphorylates PIP2 forming PIP3. PIP3 through several steps activates NOX which generate H_2O_2 which oxidizes and inactivates PTEN and other PTPs, leading to increased phosphorylation and activation of downstream Signalling. Oxidation of EGFR by H_2O_2 also potentiates its kinase activity. H_2O_2 signal is quenched by the reactivation of hyperoxidized PRDXs and oxidized PTEN by SRX and TRX respectively.*

Summarily, the binding of EGF to EGFR perpetuates downstream cellular signals through ROS-mediated activation of receptor Tyr kinases (RTKs), oxidative activation of EGFR, oxidation and concurrent reversible transient inhibition of PTPs and PTEN (which prevents futile phosphorylation/dephosphorylation cycles) leading to the activation of downstream pathways that maintain proliferation, differentiation, growth, migration, survival, angiogenesis and metastasis [44,103,106,107].

1.3.2. EGF-induced Signalling pathways

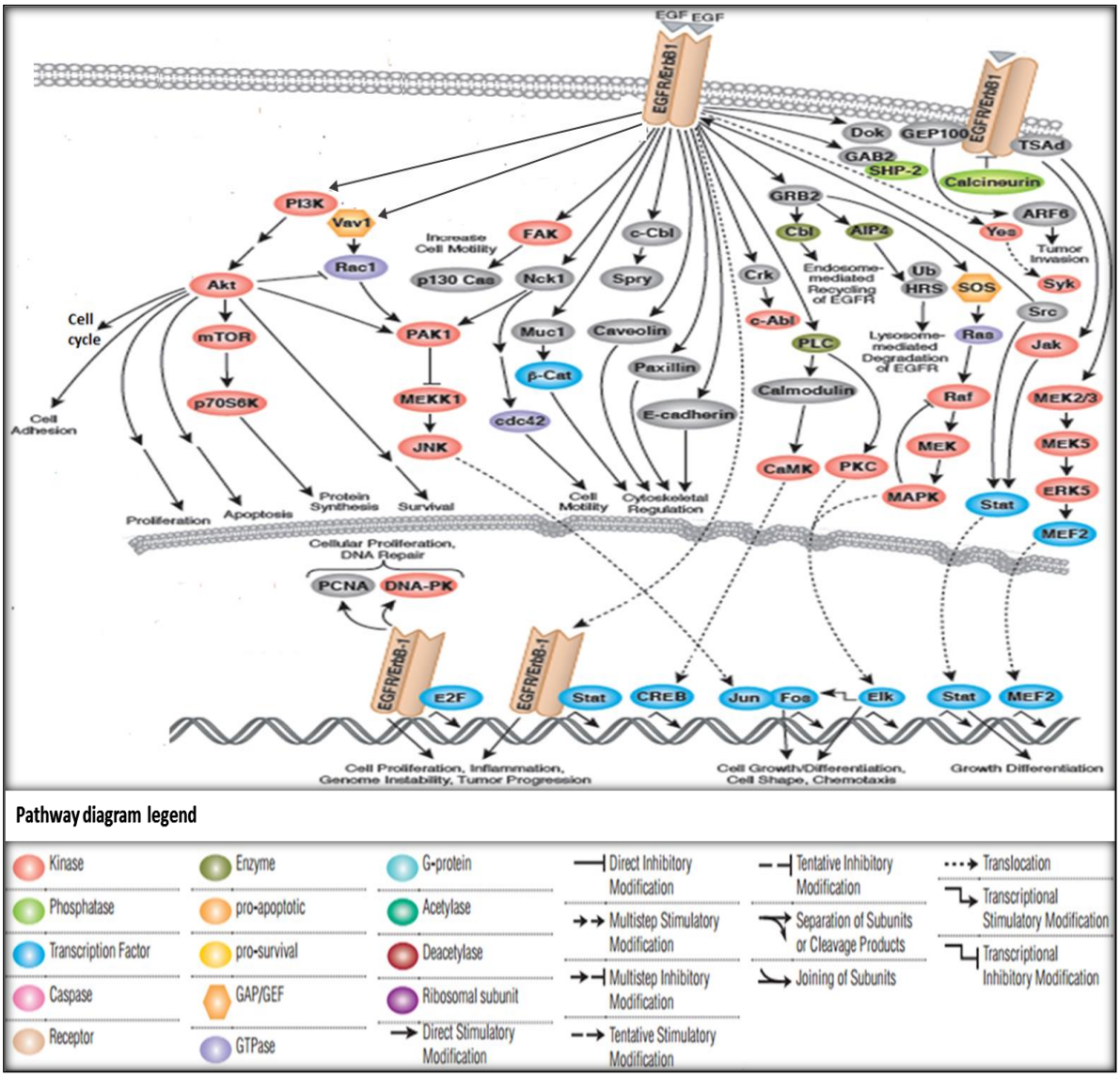

Figure 1.4: EGF-induced signalling pathways and physiological outcomes (adapted and simplified) [108]. *'Snap shot' of EGF-induced Signalling pathways and physiological consequences.*

1.3.3. EGF/EGFR involvement in cancer

Over the years, EGF/EGFR signalling has moved to the forefront of cancer research and therapeutic targeting, and rightfully so, following early reports of elevated EGFR levels in clinical samples from both human head and neck squamous cell carcinoma and lung squamous cell carcinoma [109]. Other subsequent studies confirmed the aberrant expression of

EGFR (especially the intracellular Tyr kinase domain) and its ligands in various cancers, confirming its ubiquitous implication in cancer etiology and prognosis [87,94]. The gain of function genetic alterations that have been detected in human cancers include gene amplification leading to overexpression of EGFR and EGF, kinase domain mutations which activate EGFR, in-frame deletions in the EGFR extracellular domain, and co-expression of ErbB ligands and receptors in tumors [110,111]. Each of the aforementioned alterations leads to constitutive receptor activation, which, together with the consequent ROS production and other factors downstream drive cancer initiation and progression [93]. A typical example is the observation of EGFR overexpression in about 80% of head and neck tumors correlating with poor prognosis and resistance to chemotherapy [112]. Another example is the multiple short deletions identified within the β3–αC loop of the EGFR kinase domain and also within the activation loop, P-loop, αC–β4 loop and mutations of Gly719 and Leu858 in several lung cancers which affect regulatory regions and result in enhanced kinase activity, or aberrant substrate specificity and consequent increases in ROS for downstream second messenger Signalling[113,114].

The PI3K/AKT pathway (direct target of EGFR) is one of the main constitutively active and therapeutically targeted EGF/EGFR stimulated signalling pathways in cancer which promotes growth, survival, proliferation and resistance to chemotherapy in cancer cells. Interestingly, all of the major upstream elements of this pathway have been found mutated or amplified in a plethora of cancers [68]. Gain of function mutations in *PIK3CA*, the gene encoding the p110α catalytic subunit of PI3K have been identified which enhance oncogenic PI3K signalling and, together with PIK3CA amplification, PTEN loss, AKT mutations and RTK amplification constitute frequent genomic alterations that promote tumorigenesis by upregulating the PI3K/AKT signalling axis in various tumors [68,115]. It is however noteworthy that EGF-induced growth and survival signalling is not trivial and involves complex networks with redundancies (several proteins with the same physiological effect), additive and synergistic effects which provide escape routes for cancer cells when treated with Tyr kinase inhibitors. This implies that it may be useful to target ROS levels and ROS modulators (e.g. annexin A2) in these pathways in combination with existing therapies for better outcomes.

From the above paragraphs, we can infer that EGF-induced ROS-mediated signalling addiction is common in a wide range of cancers with varied physiological effects which tend to be advantageous to cancer cells. To this respect, more research is necessary to better understand, the various components of these pathways and their alterations in tumor cells. In addition, recent evolution of resistance to existing therapeutics has created a need for novel strategies, and the direct and/or indirect control of ROS levels could be an option. Mindful of ROS scavenging ability of annexin A2 [116] and the ROS generating ability of EGF/EGFR [35], investigating the role of annexin A2 in EGF-induced signalling in cancer will provide useful insights in cancer biology and could create avenues for the development of novel more effective therapeutic regimens.

1.4. Annexin A2 (ANXA2)

The annexins belong to a family of structurally related proteins (in vertebrates: annexin A1-A13) that bind anionic phospholipids in a calcium (Ca^{2+})-dependent manner [117]. The basic structure of the annexins consist of a variable N-terminal domain containing various sites for protein-protein interactions and post-translational modifications which is responsible for the unique functions of each annexin, and a highly conserved C-terminal domain which is typically composed of four homologous domains (I - IV) (with the exception of annexin A6 that has eight of these domains), each consisting of five α-helices (A–E) with the AB and DE helices being connected by loops (AB and DE loops). One or more of these domains I–IV contain the endonexin fold (a region of homology) which is considered to be the signature amino acid sequence for the annexins, containing the calcium-binding motif (KGXGT-38 residues—D/E) and is present in at least one of the four AB loops [118]. The unique feature that distinguishes annexins from other calcium-binding proteins is their calcium-dependent reversible binding to negatively charged cellular membranes [119]. The tissue distribution and level of expression of annexins span from relatively abundant and ubiquitously expressed annexins (A1, A2, A4, A5, A6, A7, A11) to selective (annexin A3 in neutrophils and annexin A8 in the placenta and skin) or restrictive (annexin A9 in the tongue, annexin A10 in the stomach and annexin A13 in the small intestine) [120]. The annexins have also been implicated in a wide variety of intracellular membrane related functions such as exocytosis (annexins A2 and A7), endocytosis (annexins A1, A2 and A6), as membrane scaffolds (annexins A1,

A2, A4, A5 and A7), membrane reorganization (annexins A2, A3, A5 and A11), vesicular trafficking (annexins A2 and A11), regulation of ion transport across membranes (annexins A2, A4 and A6), and redox regulation (annexin A2) as well as extracellular functions such as their regulation of inflammation and apoptosis (extracellular annexin A1), fibrinolysis (annexin A2) and coagulation (annexin A5) [117,119,121].

1.4.1. Structure and functions of ANXA2

Annexin A2, (which is also referred to as p36, ANXA2, annexin II, lipocortin II, calpactin I chromobindin VIII, or placental anticoagulant protein IV), is a 36 kDa protein of the vertebrate annexin family located on chromosome15q22.2 [122,123]. It is expressed in various cells including monocytes, macrophages, endothelial cells and most cancer cells, where it is distributed mainly in the cytoplasm and plasma membrane with a small proportion in the nucleus [124,125]. ANXA2 was first identified as a substrate for the tyrosine kinase v-Src, the gene product of avian sarcoma virus which promotes cellular transformation [126]. This discovery already gave some hints for its possible involvement in cancer and various diseases as confirmed in recent research.

Like other annexins, the basic structure of ANXA2 consists of a homologous C-terminal core composed of four 70 amino acid repeats with five α helices, and a highly variable N-terminal tail. The C-terminal core contains the Ca^{2+}, phospholipid, F-actin and heparin binding sites, while the N-terminus contains the S100A10 (p11) binding site, a nuclear export sequence (Val-3-Leu-12), an acetylation site (Ser-1) as well as several phosphorylation sites such as Ser11, Tyr23 and Ser25, which can be phosphorylated by Src kinase (proto-oncogene) and protein kinase C, respectively, which influences its localization and functionality in some cell types [118,123,127] (Figure 1.5a). Furthermore, ANXA2 has been identified as a novel cellular redox regulatory protein by virtue of possessing a reactive cysteine residue (Cys-8) in the N-terminus which, together with the reducing effect of the TRX system has been proven to participate in normal and cancer cell redox metabolism and is the raison d'être of this study [116,128]. The calcium-dependent activation of ANXA2 leads to a conformational change that exposes some hydrophobic amino acids (Val-3, Ile-6, Leu-7 and Leu-10) priming the formation of a heterotetramer complex with its binding partner, S100A10, called ANXA2-

S100A10 heterotetramer (AIIt). AIIt shows a high affinity for phospholipids via the ANXA2 subunit of the complex [129]. ANXA2 has been shown to exist as a monomer, heterodimer (composed of one molecule of ANXA2 and a molecule of 3-phosphoglycerate kinase) or as a heterotetramer consisting of 2 molecules of ANXA2 bound together by a dimer of S100A10 which possesses a range of biological functions [130–132]. The ANXA2 monomer is mainly cytosolic while the other forms are predominantly membrane bound and each of the different forms have been shown to have various functions. Formation of the heterodimer results in the association of the resultant complex with the nucleus where it has been revealed to regulate DNA polymerase α [130]. The nuclear accumulation of the ANXA2 monomer has been reported to play a role in protecting cells from DNA damage during oxidative stress induced by genotoxic agents such as UV radiation, etoposide, chromium VI and gamma-radiation [133]. The most extensively studied and well documented function of AIIt on the extracellular surface is its interaction with tissue plasminogen activator (tPA) and its substrate, plasminogen, whereby it promotes the conversion of plasminogen into plasmin [130,134,135] by which it regulates fibrinolysis [122,136], facilitates tissue remodeling, degrades extracellular matrix and participates in angiogenesis [137,138]. The functions of AIIt are summarized in the schematic diagram in figure 1.5b. In addition to the aforementioned functions, Kwon *et al.*, [139] demonstrated that extracellular ANXA2 binds to and reduces the serine protease plasmin and that ANXA2 oxidized during this reaction is subsequently reduced by the TRX redox system. This study provided hints to the possibility of a redox signalling or redox regulatory function for ANXA2 and led to follow up studies of which the present study is part.

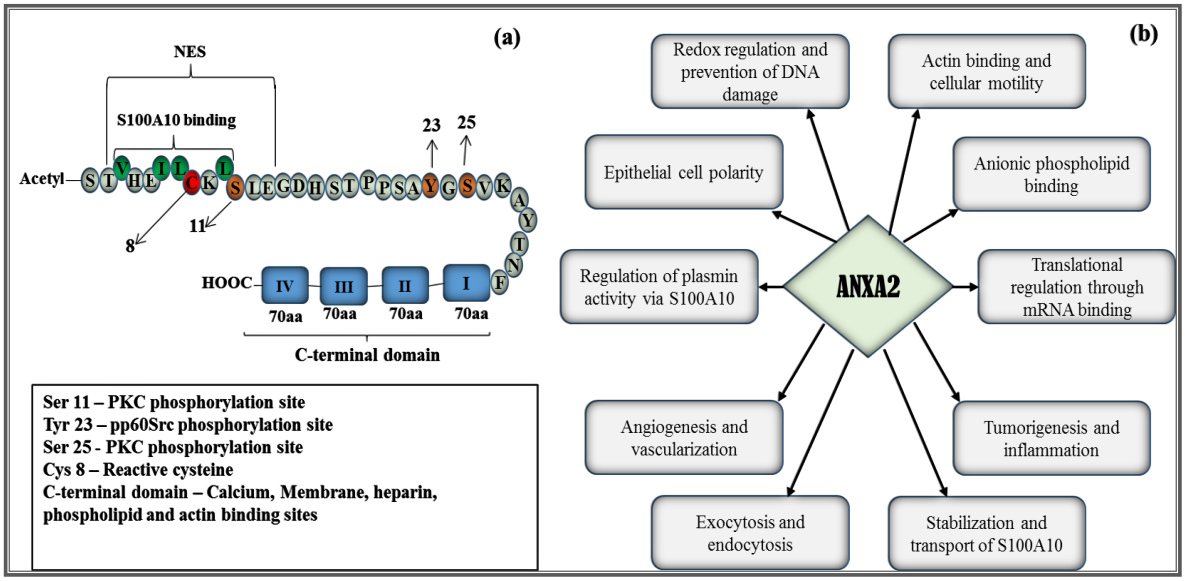

Figure 1.5: Annexin A2 domain structure (a) and functions of annexin A2 and AIIt (b) (adapted) [118]. ***(a)*** *The N-terminus of annexin A2 is the site for post-translational modifications including acetylation (S1) and phosphorylation (S11, Y23 and S25). It also contains the reactive cysteine residue (C8), the NES (V3-L12) and the hydrophobic residues, V3, I6, L7 and L10 which bind to S100A10. The C-terminal domain includes four 70 amino acid α-helices and contains binding sites for heparin, RNA, phospholipids, Ca^{2+} and F-actin.* ***(b)*** *Schematic diagram summarizing the functions of annexin A2 monomer and heterotetramer.*

1.4.2. ANXA2 in redox regulation

A unique feature which makes ANXA2 stand out among the annexins is the fact that it has redox sensitive cysteine(s) which qualify it to participate in redox homeostasis. The aforesaid role of ANXA2 in redox regulation of plasmin, coupled with the presence of redox sensitive cysteine(s) on ANXA2 that can participate in redox cycles as well as its ubiquitous and abundant cellular expression led Madureira *et al.*, [116] to postulate and confirm the cellular redox regulatory role of this protein. In the aforementioned study, Cys8 of ANXA2 was identified as the redox sensitive cysteine of ANXA2 that can be reversibly oxidized by H_2O_2 and subsequently reduced by the TRX redox system, participating in this way in several redox cycles. In addition, ANXA2 depleted cells were shown to display higher levels of ROS, increased activation of the ROS-induced pro-apoptotic kinases: JNK, p38 and Akt compared to control cells and consequent higher sensitivity to oxidative stress-mediated cell death. In addition, ANXA2 depleted cells were also significantly more sensitive to death induced by chemotherapeutic agents that produce ROS as part of their mechanism to kill cancer cells. The redox regulatory role of ANXA2 was further confirmed *in vivo* with the demonstration

of a notable increase in the oxidation of proteins in the liver and lung tissues of ANXA2- null mice compared to WT mice. In addition, a severe impairment of tumor growth was observed in tumors resulting from the subcutaneous injection of ANXA2-depleted human cancer cell lines (HT1080 and A549) in mice, compared to control cells, an effect that was reversed by the intraperitoneal administration of the antioxidant N-acetyl cysteine (NAC) to the mice during tumor development. Furthermore, *ex-vivo* experiments using human colon tumor samples showed that the cancer samples expressed significantly higher levels of the reduced form of ANXA2 in contrast to normal matched tissues and that the up-regulation of the levels of reduced ANXA2 correlated with protection from oxidation of the proteins in these tumors, indicating a possible redox regulatory role for ANXA2 in human tumors. This study led to the proposal of a mechanistic model in which H_2O_2 reacts with the Cys8 of ANXA2 resulting in its oxidation and the dismutation of H_2O_2 to H_2O. The oxidized ANXA2 is then reduced by the TRX redox system, which permits it to participate in further redox cycles [116] (Figure 1.6). Another recent study identified an important role for the ANXA2 monomer in mitigating DNA damage in response to genotoxic agents by its ROS-dependent nuclear accumulation mediated by the inactivation of ANXA2 nuclear export sequence (NES) [133]. ANXA2 is therefore part of the redox proteome and more so in cancer cells, where it will be more relevant to mitigate the possible detrimental effects of the perpetual oxidative stress.

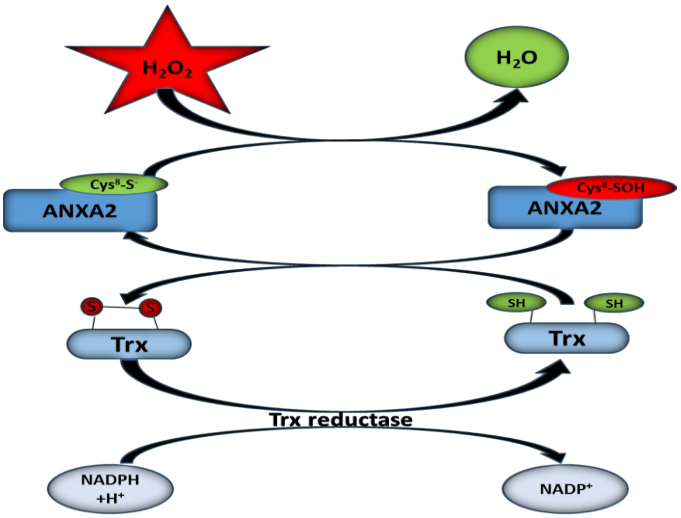

Figure 1.6: Redox mechanism for ANXA2 (adapted) [116]. *The oxidation of Cys8 of ANXA2 by H2O2 leads to the conversion of H2O2 to H2O. The oxidized ANXA2 is then reduced by the TRX redox system and can participate in additional redox cycles. A single molecule of ANXA2 can therefore participate in several redox cycles.*

1.4.3. ANXA2 in cancer

ANXA2 is aberrantly expressed in a plethora of tumors. Increased expression of ANXA2 protein and mRNA has been observed in many malignancies such as hepatocellular[140] and gastric carcinomas [141], colorectal [142], breast [143], lung [144] and pancreatic cancers [145], particularly in the more aggressive or poor prognosis phenotypes [146,147]. ANXA2 overexpression has also been positively correlated with resistance to chemotherapy and malignant progression [145,148,149].

The high expression of ANXA2 in tumor stroma and cancer cells has been associated with the enhancement of tumor cell adhesion [127]. A recent example supporting this association is the demonstration that the adhesion between breast cancer cells and endothelial cells (ECs) is mediated by interactions between ANXA2 and S100A10 in a mechanism whereby ANXA2 expressed on the surface of the cells interacts with S100A10 located on microvascular ECs and facilitates the process by which these cells form cell-cell contact with microvascular ECs which is important for tumor growth [150].

ANXA2 plays a role in the enhancement of proliferation and inhibition of apoptosis through different pathways in a number of cancer cell types. This role has been observed in a study which showed that, during oxidative stress ANXA2 depleted MCF-7, A549, HT1080 and LLC cell lines exhibited significantly greater levels of P-p38, P-Akt and slightly elevated P-JNK compared to control cells, consistent with the susceptibility of these cells to oxidative stress (ROS) induced apoptosis, indicating a ROS-mediated anti-apoptotic role for ANXA2 [116]. Another study by Madureira *et al*., demonstrated that, in response to genotoxic agents (such as gamma-radiation, UV radiation, chromium VI and etoposide), cells depleted of annexin A2 had enhanced p53 (transcription factor which regulates cell cycle arrest/apoptosis) levels, increased phosphorylation of histone H2AX (indicator of DNA damage) and increased numbers of p53-binding protein 1 nuclear foci suggesting that annexin A2 could play an anti-apoptotic role by mitigating DNA damage (a role which was confirmed to be ROS-dependent) [133]. Furthermore, other studies showed that silencing of ANXA2 expression in lung cancer (A549) cells, human hepatocellular carcinoma and breast cancer (JIMT-1 and MDA-MB-231) cells coupled to treatment with a JNK inhibitor

(SP600125) led to a significant inhibition of proliferation with a marked decrease in the percentage of cells in the S/G2+M phase of the cell cycle and an increase in p53 transcription with more cells found to accumulate in the G0/G1 phase compared to control cells (expressing normal levels of ANXA2) [151–154].

ANXA2 and AIIt also play a major role in tumor progression by enhancing neovascularization. An increase in the expression of AIIt in some tumors has been recognized as a major contributor to cancer angiogenesis *in vitro* and *in vivo* [155,156]. Recent studies using xenograft models of human breast tumors have demonstrated a marked inhibition of neoangiogenesis in the tumor microenvironment after treatment with an ANXA2 antibody, indicating the active participation of ANXA2 in the formation of new blood vessels in cancer [157]. Another study with clinical samples demonstrated the accumulation of tPA and AIIt on the surface of invasive human breast cancer cells which was positively correlated with tumor neovascularization [138].

Tumor invasiveness and metastasis is the major cause of mortality among cancer patients. The involvement of ANXA2 in tumor invasion and metastasis by interacting with the actin cytoskeleton and potential invasion-associated proteases has been reported in many late-stage human tumors [127]. A simple example was described in a study whereby the overexpression of ANXA2 was observed in the highly invasive MDA-MB-231 breast cancer cell line compared with the poorly invasive MCF-7 cell line [143]. In addition, the administration of adriamycin MCF-7/ADR to the cell cultures has been observed to increase the expression of ANXA2 with a resultant enhancement of cell proliferation and invasion, suggesting the association of ANXA2 in cancer cell invasion [158]. Also, ANXA2 knock down by siRNA or neutralizing antibodies has been observed to significantly inhibit the motility and invasion of HCC and other cancers, further indicating the contribution of ANXA2 to metastasis [152,159]. S100A10 in AIIt catalyzes the conversion of plasminogen to plasmin through its interaction with tPA and thus efficiently enhances the effects of plasmin tissue remodeling, matrix metalloproteinases and latent growth factor activation, and extracellular matrix degradation, leading to tumor progression and metastasis [122,160]. Furthermore, upregulation of ANXA2 in MCF-7 non-invasive breast cancer cells has been observed to increase the expression of c-

myc and cyclin D1 by activating the Erk1/2 signalling pathways leading to enhanced migration and invasion *in vitro* and *in vivo* [161].

From the above paragraphs, we can see that ANXA2 is an important molecule involved in regulating tumor cell adhesion, proliferation, invasion, metastasis, tumor neovasculogenesis and redox homeostasis, thus playing a crucial role in the multistep process of tumor development. However, many gaps still exist in the knowledge of the specific biochemical mechanisms and signalling pathways that bring about the aforementioned physiological effects of ANXA2 in cancer. In addition, considering the importance of ROS in the physiological outcomes of ANXA2 mentioned above, it is important for us to gain more insight into the ROS-mediated effects of ANXA2 in a bid to fill in the gaps and contribute to the identification of novel anticancer strategies.

1.5. Rationale

As mentioned earlier in this thesis, ROS are produced as a consequence of cellular metabolism and can also be produced by the cell in response to GF/GFR stimulation to function as second messengers in major signalling pathways. Even though ROS play very important roles in cell signalling events, their levels and intracellular localization must be tightly regulated within the cell (nanomolar levels required for signalling), since increased production of these highly reactive small molecules can be detrimental, leading to protein oxidation, lipid peroxidation and DNA damage, giving rise to cell death and/or tumorigenesis (in case of DNA mutagenesis). Consequently, the robust cellular redox systems play a critical role in regulating homeostasis and cellular integrity in normal and cancer cells by regulating the restricted sub-cellular location of ROS and ROS signalling events. Thanks to their ever active metabolic state and to the tumor microenvironment, cancer cells are in a perpetual state of oxidative stress and tend to up-regulate ROS scavenging systems in order to strike a balance between the ROS concentrations required for signalling and the detrimental effects of high ROS levels. Unravelling new members that contribute to cellular redox equilibrium will provide valuable tools to fight against the harmful effects of ROS in normal cells and create avenues to preferentially and specifically target cancer cells. The EGF/EGFR ligand-receptor system is ubiquitously up-regulated in most cancers whereby it enhances

downstream signalling events that favor the development of various aspects of the cancer phenotype. The binding of EGF to EGFR leads to signalling events that trigger the production of ROS (H_2O_2) as potent second messenger signal transducers which further activate other signalling pathways with various physiological outcomes. A majority of the downstream effectors of EGF/EGFR signalling are dependent on ROS, as described in previous sections of this write up. The overexpression of ANXA2 is commonly observed in a large number of cancers where it enhances the development of the main specific features of cancer. Madureira *et al.*, recently identified a novel function for ANXA2 as an antioxidant protein by virtue of its possession of a reactive Cys (Cys8) residue which participates in many redox cycles and it is recycled by the TRX system. This study also demonstrated that the redox regulatory function of ANXA2 enhances tumor growth and resistance to chemotherapy [116]. As a logical follow up of the aforesaid findings, investigating the role of ANXA2 in ligand/receptor-induced ROS-dependent signalling is necessary to further understand the redox regulatory function of ANXA2 and gain more insight in cancer cell signalling.

1.6. Objectives

1.6.1. Main objective

The main aim of this study is to investigate the role(s) of the novel antioxidant protein, ANXA2 in EGF/EGFR-induced ROS-dependent signalling pathways in cancer cells.

1.6.2. Specific objectives

In order to achieve the main objective I propose to achieve the following specific objectives:

(i) Analyse the activation of signalling pathways in ANXA2 shRNA (knockdown) versus control (scramble shRNA) cancer cells upon activation with EGF to investigate if ANXA2 plays a role in the regulation of EGF/EGFR induced signalling pathways.

(ii) Assess the oxidation of reactive Cys residues in redox-sensitive signalling proteins pathways in ANXA2 shRNA (knockdown) versus control (scramble shRNA) cancer cells upon activation with EGF.

(iii) Analyse the ROS levels of ANXA2 shRNA (knockdown) versus control (scramble shRNA) cancer cells upon activation with EGF to investigate if ANXA2 plays a role in ROS inactivation upon EGF/EGFR induced signalling.

(iv) Analyse if ANXA2 is important for cell proliferation induced by the EGF/EGFR signalling pathways.

2. MATERIALS AND METHODS

2.1. Cell culture and cell lines

All cell lines used in this study had been previously transfected with ANXA2 shRNA (knockdown and scramble). The cell lines used for this study and their respective information are summarized in table 2.1. The cells were all confirmed to be mycoplasma negative. The cells were cultured in complete Dulbecco's modified Eagle's medium[162] (HyClone™) supplemented with 10% fetal bovine serum (FBS) [SIGMA®], 100 U/ml of penicillin/streptomycin (SIGMA®) and 2 mM of L-Glutamine (SIGMA®) in a humidified incubator (*Forma™ Series II Water-Jacketed CO_2 Incubators* - Thermo Scientific) in an atmosphere of 5% CO_2 at 37º C.

Cell stocks were taken out from the -150 °C deep freezer (SANYO), left to thaw in an unstirred water bath (Clifton) at 37^0C after which they were placed in 10 ml of pre-warmed complete Dulbecco's modified Eagle's medium (DMEM) in 15 ml falcon tubes (*VWR*) and centrifuged for 5 minutes at 1000 g. The medium was aspirated to remove the dimethyl sulfoxide (DMSO) [which is toxic to the cells] and the pellets containing the cells were re-suspended in 5 ml or 15 ml of complete medium dispensed into T25 (*VWR*) or T75 (Greiner) culture flasks, respectively. Puromycin (10 mg/ml, SIGMA®) was added to each culture flask in a ratio of 1:5000 (final concentration of 2 µg/ml) in order to selectively obtain the desired phenotypes after which the flasks were placed in the humidified incubator. Cells in culture were monitored daily, and depending on the confluence of the cells observed in the flasks after microscopic examination and change in pH (orange coloration of medium), the old medium with puromycin was aspirated and replaced or the cells were passed. It is noteworthy that all manipulations of cells were performed in sterile Laminar flow hoods *Microflow® Biosafety Cabinet Class II* (*Telstar*) following established tissue culture standard operating procedures.

Table 2.1: Cell lines, origins, characteristics and suppliers.

Cell line	Origin and characteristics	Producer
MDA-MB-231	Human adenocarcinoma of the breast (derived from metastatic site: pleural effusion)	ATCC®
MDA-p36 KD-3	MDA-MB-231 cells expressing ANXA2 shRNA-3 (obtained by cloning of the dsDNA oligo 5'-GATCCCCCCTGGTTCAGTGCATTCAGTTCAAGA GACTGAATGCACTGAACCAGGTTTTTA-3' and 5'-AGCTTAAAAACCTGGTTCAGTGCATTCAGT CTCTTGAACTGAATGCACTGAACCAGGGGG-3') in a pSUPER-retro-puro vector as "backbone" [OligoEngine])	Our Laboratory
MDA-p36 KD-4	MDA-MB-231 cells expressing ANXA2 shRNA-4 (obtained by cloning of the dsDNA oligo 5'-GATCCCCGTGCATATGGGTCTGTCAATTCAAGAGA TTGA CAGACCCATATGCACTTTTTA-3' and 5'-AGCTTAAAAAGTGCATATGGGTCTGTCAATCTCTTG AATTGACAGACCCATATGCACGGG-3' in a pSUPER-retro-puro vector as "backbone" [OligoEngine])	Our Laboratory
MDA-p36 Scramble	MDA-MB-231 cells expressing ANXA2 scramble (control) shRNA (obtained by clonining of the dsDNA oligo 5'-GATCCCCGTGCATATGGGTCTGTCCATTAGAGAGA TTGACAGACCCATATGCACTTTTTA-3' and 5'-AGCTTAAAAAGTGCATATGGGTCTGTCAATCTCTCT AATGGACAGACCCATATGCACGGG-3' in a pSUPER-retro-puro vector as "backbone" [OligoEngine])	Our Laboratory
293T	Human embryonic kidney epithelial cells	ATCC®

2.1.1. Passaging of cells, preparation of plates, treatments and stocks

For passaging of cells, after examination under the microscope (Zeiss, primo vert), culture medium was aspirated, followed by washing of the cells with 5 ml (T75) or 2 ml (T25) of Dulbecco's Phosphate Buffered Saline (without Ca^{2+} or Mg^{2+} - LONZA). The cells were then

detached by 3 – 5 minutes incubation with 3 ml (T75) or 1 ml (T25) of 0.25% trypsin-EDTA (SIGMA®) in a humidified atmosphere of 5% CO_2 at 37º C. After observing complete detachment of the cells under the microscope for, 2.7 ml (T75) or 0.8 ml (T25) of the mixture was dispensed in to 15 ml tubes for further procedures, while the remaining volume was placed back in culture with fresh medium and 1 µg/ ml puromycin.

The tubes were centrifuged at 1000g for 5 minutes and the resultant pellets re-suspended in 4 ml of complete DMEM and homogenized. Cells were then counted by pipetting out 10 µL of the suspension and placing it into a 0.0025 mm^2 Neubauer counting chamber (Hausser scientific) and counting the number of cells present in four squares of 1 mm^2. The number of cells per ml were determined using the following formula:

Average number of cells per square × dilution factor × 10^4 = number of cells per ml.

In order to perform the signalling experiments with EGF stimulation, 60 mm sterile plates (*VWR*) were prepared, each with a final volume of 3 ml consisting of the cell suspension (usually about 5 x 10^5 cells/plate) and complete DMEM. The plates were incubated in a humidified atmosphere of 5% CO2 at 37º C for 24 to 48 hours, after which they were usually starved overnight (to minimize any serum-dependent and external effects) by taking out the complete DMEM, washing with phosphate buffered saline (PBS) and replacing it with DMEM without FBS. After checking the cells under the microscope and confirming equivalent confluency between the cell lines, the cells were treated with 50 ng/ml of EGF (*biorbyt*™ - orb49362) at various time points.

Cell stocks were made for each of the cells used in this study, usually from the first selection after putting the cells in culture. In order to freeze the cells, the pellets (obtained as explained above) were re-suspended in a cryoprotective freezing solution (90 % FBS + 10 % DMSO), properly homogenized and pipetted into 2 ml freezing tubes (*VWR* and SIGMA®) with a cell density of 2-3 x 10^6 cells/ml. The freezing tubes were then wrapped in paper and rapidly stored in the -80°C deep freezer (New Brunswick Scientific) for 24 hours, after which they were transferred to -150 °C for long term storage.

2.2. Preparation of protein lysates and quantification

In order to obtain the lysates containing all proteins from the cells, either not treated (NT) or after treatment with EGF for different time points (time courses), the medium from each 60 mm plate was first discarded, followed by thorough washing with 1 ml of PBS (with Ca^{2+} and Mg^{2+}) after which the PBS was pipetted out. 100 to 150 µL of lysis buffer (50 mM Tris-HCL pH 7.5, 150 mM NaCl, 1 % Nonidet® P-40 substitute, 1 % sodium deoxycholate, 1 mM EDTA, 2 mM EGTA, 10 mM sodium fluoride (NaF), 1 mM sodium ortovanadate (Na_3VO_4), 0.5 % protease inhibitors [SIGMA®, ref: P2714-1BTL]) was added to each plate followed by scrapping to lyse the cells. The resultant lysates were pipetted into priory labeled sterile 1.5 ml *Eppendorf* tubes, left for 10 minutes on ice, and centrifuged at maximum speed (15000 g) in a refrigerated centrifuge (Heraeus™ Fresco 17 Centrifuge [Thermo-Scientific]) for 15 minutes at 4 °C (precipitation of non-soluble components). After centrifugation, the supernatants were pipetted into fresh sterile *Eppendorf* tubes and stored at -80 °C. It is important to note that after treatments, all procedures were performed on ice (4 °C) to minimize protein degradation.

Prior to running gels and western blots, the protein concentration in each sample was determined. The samples were prepared using the *Pierce*® bicinchoninic acid (BCA) protein assay kit (Thermo-scientific) according to the manufacturer's instructions. The BCA protein assay is based on the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (Biuret reaction). This reduction permits the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) by BCA. The stoichiometric reaction involves the chelation of two molecules of BCA with each Cu^{+} forming a water soluble purple colored BCA/copper complex which exhibits a strong linear absorbance at 562 nm which is directly proportional to protein concentrations. Prior to each set of measurements, a linear calibration curve was established for a range of concentrations of interest (usually [0.4 – 1] µg/µl) using bovine serum albumin (BSA) standards provided as part of the kit. A reference solution containing milli Q water was also used for each set of measurements and the samples were diluted in a ratio of 1:10 in order to keep the concentrations within the calibration range. After proper blanking and calibration, the protein concentration in each sample was determined using the

NanoDrop 2000c UV-Vis Spectrophotometer (Thermo-Scientific) at 562 nm following the manufacturer's guidelines.

2.3. Western blotting

For western blot analysis loading fractions containing 20 µg/20 µl of the extracted protein samples in 1x loading buffer (with or without β-mercaptoethanol), were subjected to SDS-PAGE. The gels were prepared using the stock solutions of 40% de acrilamide:bisacrilamide (29:1) (AppliChem®), 1M Tris (pH 8,8), 10% SDS (AppliChem®), 25% ammonium persulfate (Sigma®), TEMED (AppliChem®) and *milliQ* water. Appart from the aforementioned constituents, the stacking gel was prepared using 1M Tris (pH 6,5) with a final concentration of 5% of acrilamide:bisacrilamide (29:1).Considering the expected sizes of the proteins of interest in this study, running gels with 10% and 12% of acrylamide:bisacrilamide (29:1) were used (details in appendix 4, page 75). The gels were run using the *Mini-Protean* (BioRad®) electrophoresis system and power source (*PowerPac*™ Basic power supply [BioRad®]) according to the manufacturer's instructions, completely submerged in electrophoresis running buffer (details in appendix 5, page 76) with 20 µl of sample (20 µg of protein) loaded into each well and 2 µl of the molecular weight marker (*nzytech*, batch-13121) in the first well. An initial voltage of 100 V (0.05 – 0.08 A) was used to get the proteins stacked into the running gel, after which the voltage was increased to 120 – 130 volts maintained at 0.05 – 0.08 A. The usual running time was about 80 minutes.

The separated proteins (in bands) within each gel were transferred on to 0.2 µm nitrocellulose membranes (BioTrace™ NT [Pall Corporation®]) using the standard sandwich transfer procedure [163], completely submerged in blotting buffer (details in annex) with ice packs, at 100 V for 70 minutes. The membranes were then blocked with 5% skimmed milk in Tris buffered saline-tween 20 (TBS-T) for a minimum of 30 minutes (to prevent non-specific antibody binding, considering the high non-specific affinity of nitrocellulose membranes for proteins) at room temperature with mild shaking on the plate mixer (*VWR*® Mini Blot Mixer). After blocking, the membranes were incubated overnight at 4 °C (with mild shaking) with l primary antibody (in TBS-T with BSA or milk and 0.05% azide) specific for the target

proteins. After the first incubation, the membranes were washed four times each with 5 – 10 ml of TBS-T (5 minutes per wash) with shaking after which they were incubated with appropriate horse radish peroxidase (HRP)-conjugated secondary antibodies (dilution of 1: 5000 in TBS-T) for at least 1 hour and washed five times with 5 – 10 ml of TBS-T (5 minutes per wash) with shaking. All antibodies used in this study are indicated in table 2.2. The resultant protein bands were visualized using an enhanced chemiluminescence (ECL) solution (details in appendix 6, page 77) and images were obtained using a Molecular Imager® ChemiDoc™ XRS System (BioRad).

Table 2.2: Primary and secondary antibodies used in this study

Primary antibodies		
Antibody	**Description and reference**	**Supplier**
p36 (annexin A2)	Mouse IgG;	Made in house
p-AKT	Rabbit IgG; sc-7985	Santa Cruz Biotechnology
Total AKT	Goat IgG; sc-1618	Santa Cruz Biotechnology
p-ERK 1/2	Goat IgG; sc-16982	Santa Cruz Biotechnology
Total ERK 1/2	Mouse IgG; sc-135900	Santa Cruz Biotechnology
PTEN	Mouse IgG; sc-7974	Santa Cruz Biotechnology
PRDX II	Goat IgG; sc-23967	Santa Cruz Biotechnology
Actin	Goat IgG; sc-1615	Santa Cruz Biotechnology
Secondary antibodies		
Anti-mouse	Donkey IgG-HRP; sc-2314	Santa Cruz Biotechnology
Anti-goat	Bovine IgG-HRP; sc-2384	Santa Cruz Biotechnology
Anti-rabbit	Goat IgG-HRP; sc-2004	Santa Cruz Biotechnology

2.4. Biotinylated Iodoacetamide (BIAM) assay

BIAM assays are widely used to asses modification (oxidation) of reactive Cys thiols based on the selective alkylation of free (non-oxidized) thiols and detection of alkylated proteins. Here, biotin conjugated iodoacetamide (BIAM) reacts with free reactive Cys thiolate anions

(reduced form) yielding biotin-carbamidomethylated cysteines, by bimolecular nucleophilic substitution (S_N2) reactions and the biotin signal is subsequently measured by standard immunoblotting using streptavidin-conjugated-HRP [164,165]. The loss or absence of the biotin signal is proportional to the degree of thiol oxidation [164].

After treating the cells with EGF, lysis and protein extraction, 150 - 200 µL of each lysate was pipetted into fresh *Eppendorf* tubes containing 1 µL (200 µM) of BIAM, after which they were incubated for 30 minutes at room temperature, followed by 30 minutes incubation at 4 °C. The reaction was then stopped by adding 1 µL IAM (200 µM) after which the lysates were incubated for 15 minutes at 4 °C, centrifuged for 15 minutes at maximum speed (15000 g) at 4 °C, and the supernatants were transferred into fresh *Eppendorf* tubes. 20 µg of each protein lysate was then analyzed by western blotting (see section 2.3) with HRP-conjugated streptavidin.

2.5. Determination of intracellular ROS levels

2′,7′ dichlorofluorescein diacetate (DCFH-DA) has been widely used as a potent marker for oxidative stress, and has been suggested to be a good indicator of the overall redox status of the cell [166]. This hydrophobic non-fluorescent molecule performs its function by penetrating rapidly into the cell, where it is hydrolyzed by intracellular esterases to yield the DCFH molecule which can be oxidised (by ROS) to its fluorescent 2-electron product 2′,7′dichlorofluorescein (DCF) that can be quantified by a fluorometer plate reader at Exc 492 nm and Em 530 nm [166]. Here, the amount of fluorescence observed is directly proportional to amount of ROS present in the cells.

For the ROS assays, 2 X 10^4 cells were set up in each well of a 96 wells plate, after which they were incubated in complete medium for 24 – 48 hours at 37 °C, 5% CO_2. After microscopic examination of the wells, the medium was removed and the cells in each well were washed with 100 µl of PBS with Ca^{2+} after what the PBS was removed. Fresh PBS with Ca^{2+} containing 200 µM DCFH-DA reagent was then added (100 µl/ well) followed by incubation for 15-30 minutes at 37°C, 5% CO_2 in the dark (DCF-DA is light sensitive) after which the cells were treated with EGF (50 ng/ml). Kinetic time course fluorescence

measurements were done using a fluorometer plate reader (TECAN – Infinite M200) at excitation - 492 nm and emission – 530 nm.

2.6. Cell proliferation assay

The evaluation of cell proliferation upon EGF stimulation in ANXA2 depleted and scramble cells was done using the CellTiter 96® non-radioactive Cell Proliferation Assay (Promega). This assay is based on the cellular mitochondrial reductase-catalyzed conversion of a tetrazolium salt (3-[4,5-dimethylthiazol-2-yI]-2,5-diphenyltetrazolium bromide), usually called MTT, into a formazan product ([*E,Z*]-5-[4,5-dimethylthiazol-2yI]-1,3-diphenylformazan) that is easily detected by spectrophotometry using a 96-well plate reader [167,168]. Here, the 570 nm absorbance reading is directly proportional to the number of viable cells.

For the MTT assays, 10^4 cells were set up in each well of a 96 wells plate and incubated in complete medium for 24 hours at 37°C, 5% CO_2. After microscopic examination of the wells, the medium was removed and the cells in each well were washed with 100 µl of PBS after what the PBS was removed. The cells were then starved overnight by incubation at 37°C, 5% CO_2, with DMEM without FBS and treated with EGF (50 ng/ml) for 6 – 24 hours. Five hours to the end of the required treatment time, 8 µL of the premixed optimized dye solution was added to each well, followed by 4 hour incubation at 37°C, 5% CO_2 (for MTT conversion to formazan product). 40 µL of the solubilization solution/stop mix was then added to the wells to solubilize the formazan product (purple coloration observed) and the absorbance at 570 nm was recorded using a 96-well plate reader (TECAN – Infinite M200).

2.7. Statistical analysis

The data obtained from the MTT (absorbance) and DCFH-DA (fluorescence) assays were transferred to spreadsheets (Microsoft excel 2013 ®) analyzed using the Student-t test for comparison of means at a 95 % confidence level. P-values below 0.05 were considered statistically significant.

3. RESULTS

3.1. Analysis of activation of signalling pathways upon treatment with EGF

In order to analyze the activation of signalling pathways in ANXA2 shRNA (knockdown) versus control (scramble shRNA) cancer cells upon treatment with EGF at different time points (time course), MDA-MB-231 cells (expressing ANXA2 shRNA or scramble shRNA) were cultured in 60 mm sterile plates and treated with 50 ng/ml of EGF (15 nM) at various time points followed by lysis to extract the protein content of these cells and western blot analysis with antibodies for a variety of target proteins (figures 3.1 and 3.2).

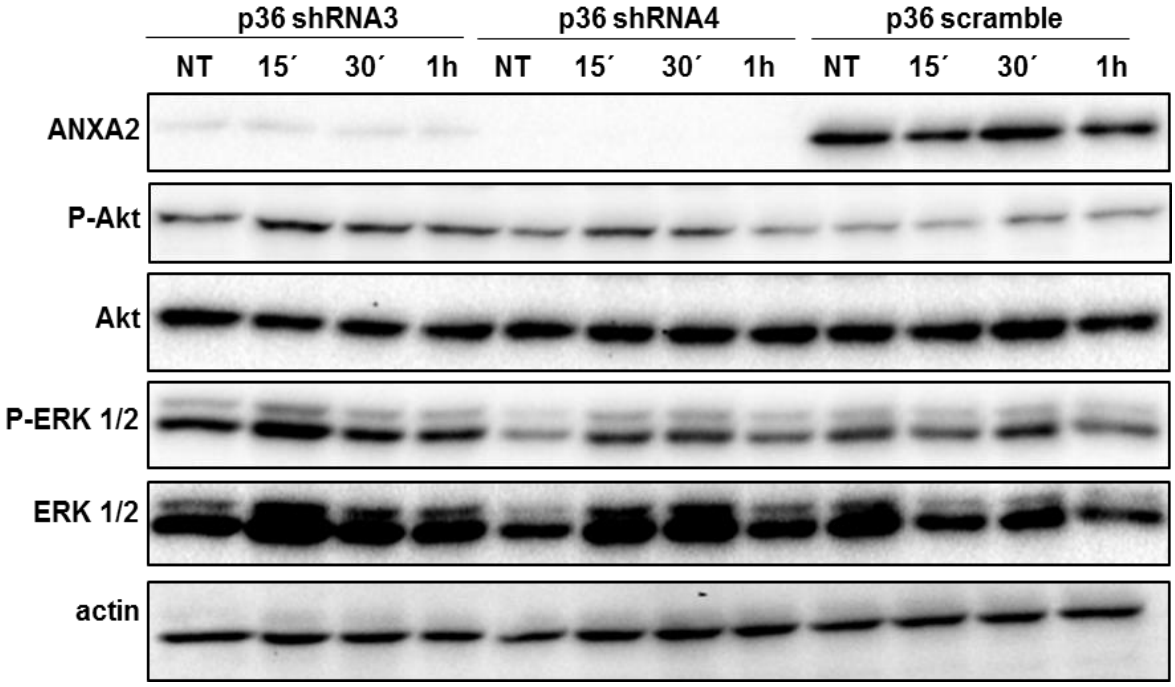

Figure 3.1: ANXA2 knockdown cancer cells show enhanced activation of the PI3K signalling pathway in response to EGF treatment compared to control cells. *MDA-MB-231 cells (ANXA2 p36 shRNA 3 – left panel, ANXA2 p36 shRNA 4 – middle panel and ANXA2 p36 shRNA scramble – right panel) were treated with 50ng/ml EGF for the indicated times followed by western analysis with the above mentioned antibodies.*

The results obtained indicate that ANXA2 knockdown cells show enhanced activation of the PI3K signalling pathway in response to EGF treatment compared to control cells. This can be observed from the enhanced phosphorylation/activation of Akt (P-Akt) in the ANXA2

depleted cells as compared to the control cells upon EGF treatment. In order to further investigate and confirm the aforementioned observations, western blot analysis with antibodies for PTEN (the main inhibitor of the PI3K pathway), PRDX II (a redox regulatory protein) and the previously mentioned signalling proteins were done (figure 3.2).

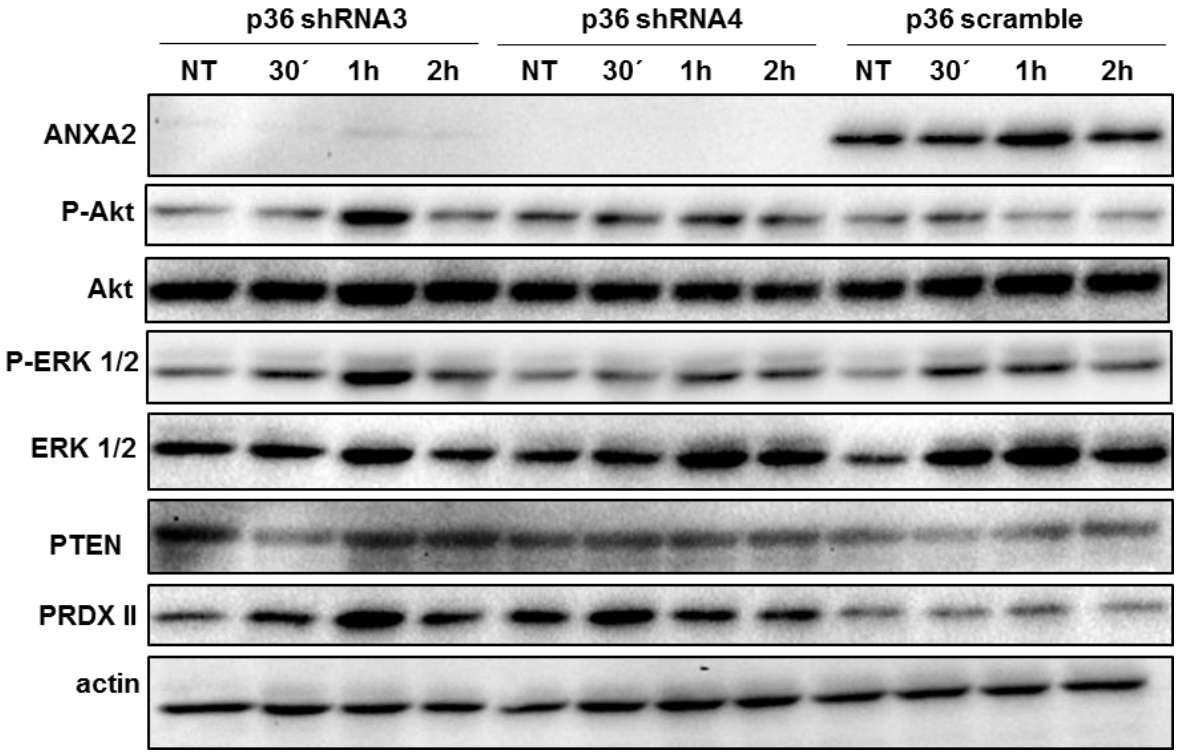

Figure 3.2: ANXA2 knockdown cancer cells show similar levels of PTEN and upregulation of PRDX II expression in response to EGF treatment compared to control cells. . *MDA-MB-231 cells (ANXA2 p36 shRNA 3 – left panel, ANXA2 p36 shRNA 4 – middle panel and ANXA2 p36 shRNA scramble – right panel) were treated with 50ng/ml EGF for the indicated times followed by western blots with the above mentioned antibodies.*

The results in figure 3.2 indicate that ANXA2 knockdown and control MDA-MB-231 cells treated or not treated with EGF show identical levels of PTEN expression. Interestingly, ANXA2 knockdown seems to lead to the upregulation of the ROS scavenger protein, PRDX II in the MDA MB 231 cells. This might be to compensate for the loss of the ANXA2 redox regulatory protein. Considering the inhibitory phosphatase role of PTEN in the PI3K pathway, its inactivation is required for the activation of the pathway. In order to gain more insight into the activity of PTEN in MDA MB 231 cells upon EGF stimulation, a non-reduced

SDS-PAGE was done followed by western blot analysis with antibodies for PTEN and ANXA2 (figure 3.3).

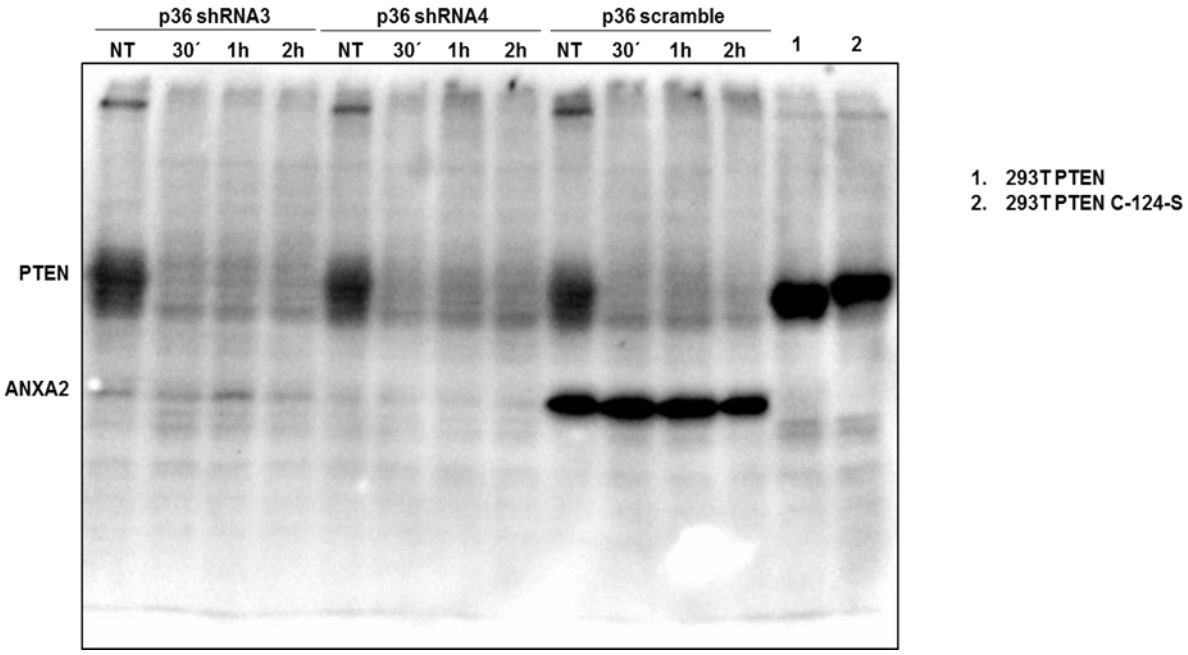

Figure 3.3: ANXA2 knockdown and control cells indicate a change in conformation of PTEN in response to EGF treatment. *MDA-MB-231 cells (ANXA2 p36 shRNA 3 – left panel, ANXA2 p36 shRNA 4 – middle panel and ANXA2 p36 shRNA scramble – right panel) were treated with 50ng/ml EGF for the indicated times followed by non-reduced SDS-PAGE and western blot analysis with the above mentioned antibodies. 293T cells with wild type and mutant (C–124-S) PTEN were also used as controls (last two lanes on the right).*

As explained earlier in this thesis, stimulation of cells with EGF leads to the production of ROS for downstream signalling. The results in figure 3.3 indicate the possibility of changes in conformation of PTEN upon EGF treatment which lowers the affinity of the monoclonal PTEN antibody used for this experiment as demonstrated by the significantly weaker signal upon EGF stimulation compared to non-treated cells. The presence of bands in the 293T cell panels shows that the antibody does not bind to the reactive Cys residue of PTEN (Cys124) [44]. It is therefore possible that the treatment of the cells with EGF leads to oxidation and conformational changes (disulfide bond formation) in PTEN which could inactivate it. The fact that under reducing conditions (figure 3.2.) the affinity of the PTEN antibody did not change if we compare the non-treated samples with the EGF treated samples, indicates that

the difference observed in non-reducing conditions is most likely due to conformational changes that occur via disulfide bond(s) formation upon EGF treatment.

In summary, the above mentioned results indicate that ANXA2 knockdown cells show enhanced activation of the PI3K pathway and possible compensatory upregulation of PRDX II expression compared to control cells.

3.2. Assessment of oxidation of reactive Cys residues in redox-sensitive proteins upon treatment with EGF

BIAM assays were performed to assess the EGF induced ROS-mediated oxidation of reactive Cys residues in redox sensitive proteins in ANXA2 knockdown and control cells. Here, cell lysates were labeled with 200 µM of BIAM and subjected to SDS-PAGE and western blotting with a streptavidin probe (See section 2.4). A decrease in BIAM labeling of redox sensitive proteins, as observed by streptavidin blot analysis indicates oxidation of reactive Cys by the EGF induced ROS. The results obtained indicate that ANXA2 knockdown and control cells show similar redox profiles upon BIAM labelling after EGF treatment (figure 3.4). Overall there was no significant increase in total redox sensitive protein oxidation upon treatment with EGF. This is probably due to the nature of ROS dependent signaling which is mediated in the order of nanomolar concentrations of H_2O_2 (low concentration) and is very localized (within the area of signal transduction). For this reason the overall redox status of the cell does not seem to change significantly, as probably only a small fraction of redox sensitive proteins are being oxidized during EGF signaling.

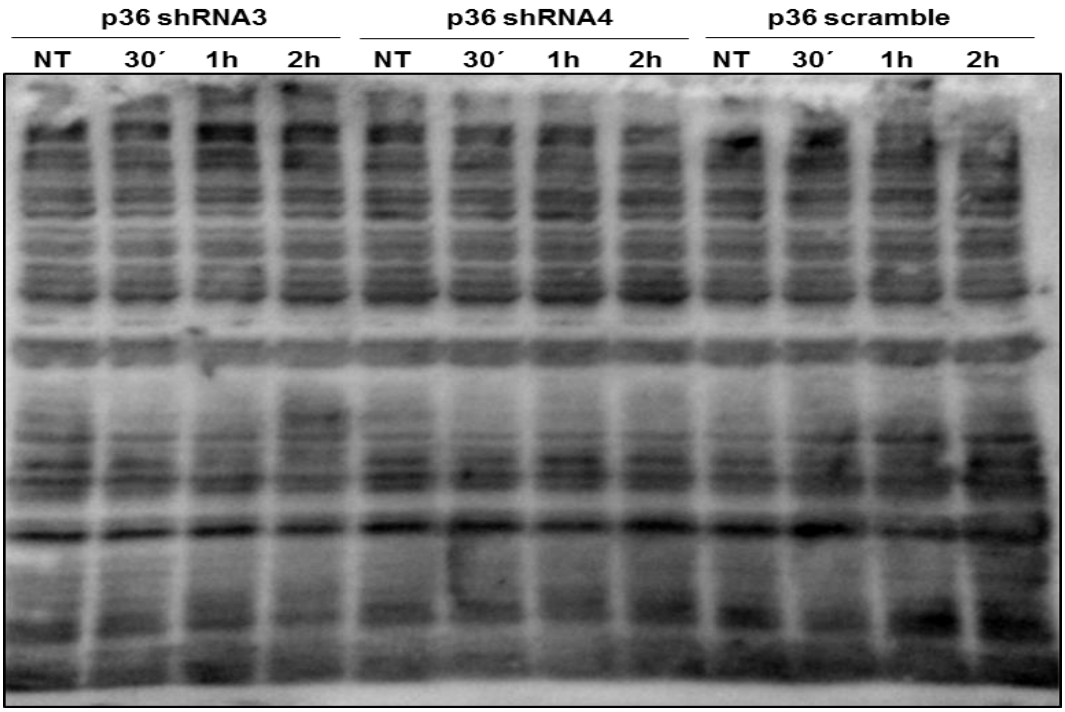

Figure 3.4: ANXA2 knockdown and control cells show similar redox profiles upon BIAM labelling after EGF treatment under non-reduced conditions. *MDA-MB-231 cells (ANXA2 p36 shRNA 3 – left panel, ANXA2 p36 shRNA 4 – middle panel and ANXA2 p36 shRNA scramble – right panel) were treated with 50ng/ml EGF for the indicated times followed by labelling with 200 µM of BIAM, SDS-PAGE and western blot analysis with streptavidin probe.*

3.3. Analysis of ROS levels in ANXA2 knockdown versus control MDA-MB-231 cancer cells upon treatment with EGF

In order to investigate if ANXA2 plays a role in ROS inactivation upon EGF treatment, the ROS levels of ANXA2 knockdown versus control cancer cells were analyzed. Here, the intracellular ROS levels in non-treated cells and cells treated with 50 ng/ml of EGF for 15 minutes were measured using the fluorescent probe, 2′,7′ dichlorofluorescein diacetate (DCFH-DA)[see section 2.5]. As expected, treatment of MDA MB 231 cells with EGF led to enhanced levels of ROS in the ANXA2 depleted and control cells (figure 3.5). These results also showed that ROS levels were significantly higher in ANXA2 knockdown cells compared to control cells after treatment with EGF for 15 minutes indicating that ANXA2 plays a role in ROS inactivation upon EGF induced signalling (figure 3.5). Comparison of the levels of ROS between the ANXA2 depleted and control cells revealed a significant increase in ROS levels in ANXA2 p36 knockdown-3 cells ($P = 0.000042$) and ANXA2 p36

knockdown-4 cells (P = 0.042) compared to control cells confirming the aforesaid observation.

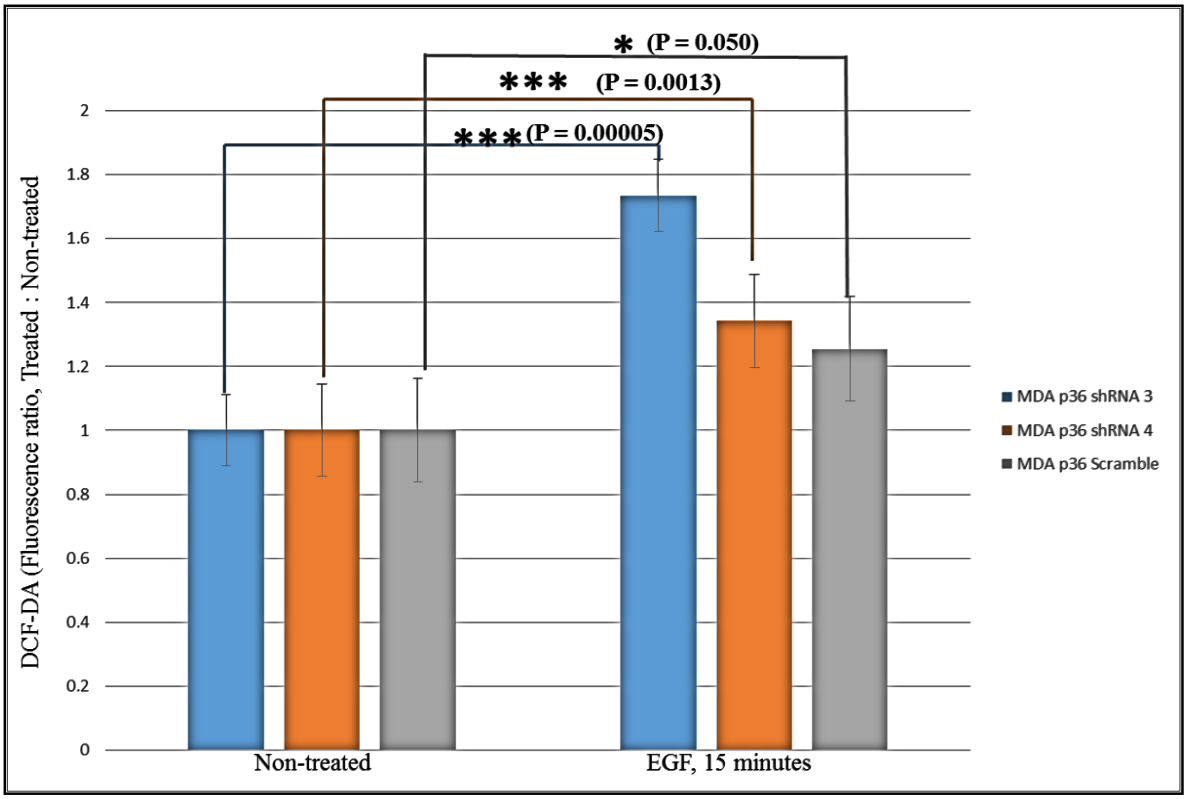

Figure 3.5: ANXA2 knockdown cells accumulate higher levels of ROS upon EGF stimulation. *MDA-MB-231 cells (ANXA2 p36 shRNA 3 – blue, ANXA2 p36 shRNA 4 – orange and ANXA2 p36 shRNA scramble – grey) were serum starved for 16 hours and then incubated with 200 µM of DCFH-DA reagent, followed by treatment with 50 ng/ml EGF for 15 minutes. Fluorescence was measured using a fluorometer plate reader (Exc. 492 nm, Em. 524 nm). Data are represented as fold induction of ROS between non-treated and treated cells (N = 6). P-values represent the difference in ROS levels between non-treated and EGF treated cells at 95 % significance level.*

3.4. Evaluation of the role of ANXA2 in cell proliferation induced by the EGF/EGFR signalling pathways

In order to analyse if ANXA2 is important for cell proliferation induced by the EGF/EGFR signalling pathways, the rate of proliferation of ANXA2 depleted and scramble (control) MDA MB 231 cells was measured using the CellTiter 96® non-radioactive Cell Proliferation MTT Assay (see section 2.6). Here the rate of proliferation of ANXA2 depleted and control cells was determined upon treatment with 50 ng/ml of EGF for 24 hours and compared with proliferation rates in non-treated cells (figure 3.6). A comparison of the proliferation rates

between the non-treated and EGF-treated ANXA2 depleted cells indicated a significant increase in the rate of proliferation upon EGF treatment (P-values shown on figure 3.6), while the control cells showed a non-significant increase in proliferation rate upon treatment with EGF (P = 0.40). A comparison of the rate of proliferation between the ANXA2 depleted and control cells revealed a significant increase in the proliferation rate of ANXA2 p36 knockdown-3 cells (P = 0.020) and ANXA2 p36 knockdown-4 cells (P = 0.0017) compared to control cells. These results show that ANXA2 knockdown cells have higher rates of proliferation upon EGF treatment compared to control cells.

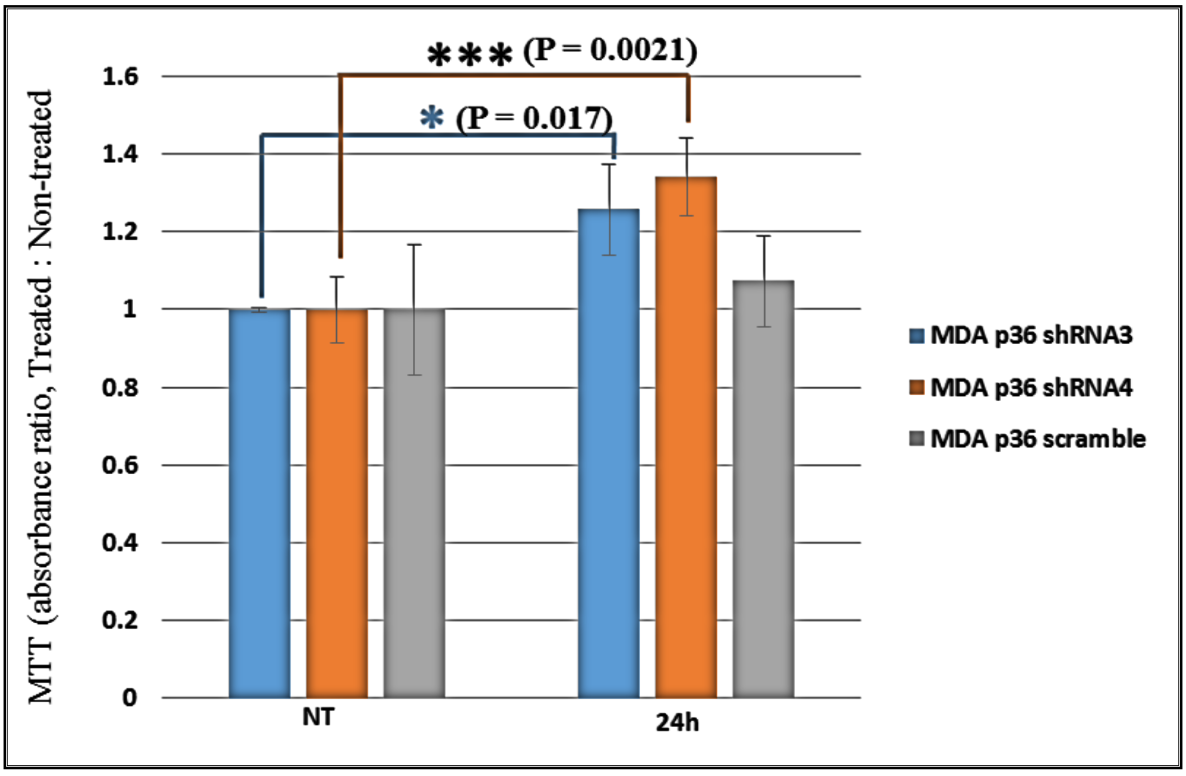

Figure 3.6: ANXA2 knockdown cells have a higher proliferative rate upon EGF treatment compared to control cells. *MDA-MB-231 cells (ANXA2 shRNA 3 – blue, ANXA2 shRNA 4 – orange and ANXA2 shRNA scramble – grey) were serum starved for 16 hours followed by mock treatment or treatment with 50 ng/ml EGF for 24 hours. Absorbance (λ = 570 nm) was measured after a tetrazolium reduction cell proliferation assay. Data are represented as folds of increase in proliferation rates between mock treated and treated cells (N = 6). P-values represent the difference in ROS levels between non-treated and EGF treated cells at 95 % significance level.*

4. Discussion

Our laboratory recently identified ANXA2 as a new redox regulatory protein in cells that plays a major role during oxidative stress and particularly in tumorigenesis [116]. As a follow up to this research, the present study aimed at investigating the role of ANXA2 in GF/GFR induced ROS mediated signalling. The choice of EGF/EGFR is as a result of the fact that the binding of EGF to its cell surface receptor leads to downstream evens that transiently increase the intracellular levels of H_2O_2 for downstream signalling events (activation of kinases and deactivation of phosphatases) [35,103].

As stated earlier in this thesis, the PI3K pathway plays a key ROS-dependent role in cell proliferation and survival in response to EGF stimulation and is one of the most mutated pathways in cancer [42,68]. The results of this work show, for the first time, that ANXA2 knockdown in cancer cells leads to enhanced activation of the PI3K pathway (figure 3.1) in response to EGF treatment. This study also showed that ANXA2 depleted cells have higher levels of ROS and proliferation rates upon treatment with EGF. Together these results suggest that the stimulation of ANXA2 depleted cancer cells with EGF leads to enhanced H_2O_2 levels that over activate PI3K signalling compared to control cells. As a result of this, the ANXA2 depleted cells show higher proliferative rates in response to EGF treatment. ANXA2 therefore plays a redox regulatory role via modulation of the PI3K pathway. The phosphatase, PTEN, is the main inhibitor of the PI3K pathway and contains reactive Cys residues in its catalytic domain that can be readily oxidized by H_2O_2 inactivating its phosphatase function [104]. The inactivation of PTEN is a pre-requisite for PI3K signalling. Our results show that the treatment of ANXA2 knockdown and control cells with EGF does not significantly alter the expression of PTEN (figure 3.2). This demonstrates that the inactivation of PTEN upon EGF treatment is not as a result of a decrease in expression, but most probably due to the EGF induced ROS mediated oxidation of the reactive Cys residues in its catalytic site. These results are in agreement with earlier studies which demonstrate a reversible inactivation of PTEN by H_2O_2 as a regulatory mechanism for PI3K signalling [43,103,104]. Further *in vitro* and *in vivo* experiments with different cancer cell types are ongoing in our laboratory to confirm the aforementioned results.

The results obtained with western blots for PTEN upon treatment of the cells with EGF under non-reduced conditions indicate the possibility of changes in conformation of PTEN upon EGF treatment which lowers the affinity of the monoclonal PTEN antibody used for this experiment (figure 3.3). This result, coupled to the fact that in reducing conditions the affinity of the PTEN antibody did not change in the non-treated samples compared to EGF treated samples (figure 3.2.) indicates that the difference observed in non-reducing conditions is most likely due to conformational changes that occur via disulfide bond(s) formation upon EGF treatment. Therefore the treatment of the cells with EGF leads to an increase in intracellular ROS levels (more so in ANXA2 depleted cells) which may lead to oxidation and conformational changes in PTEN which could inactivate it and by so doing, enhance the ROS mediated activation of PI3K signalling. However, western blot analysis is required with monoclonal antibodies specific for the catalytic site reactive Cys residues of PTEN to validate these assertions. Further research is ongoing in our laboratory to determine the interactions between ANXA2 and PTEN in a bid to better understand the redox regulatory functions of ANXA2.

PRDX II is a highly abundant antioxidant protein present in the cytosol of several cell types [24]. In order to investigate the role of PRDX II in EGF induced signalling in ANXA2 depleted and control cells, western blotting with an antibody for PRDX II was done (figure 3.2). Interestingly, the results obtained demonstrate, for the first time, that MDA MB 231 ANXA2 knockdown cells show higher levels of PRDX II compared to control cancer cells. This result suggests that the cells might be responding to ANXA2 knockdown by increasing the expression of another ROS scavenger, PRDX II to compensate for the loss of ANXA2 redox regulatory function. Further investigation of the possible cross talk between ANXA2 and PRDX II is currently ongoing in our laboratory.

Investigation of the role of ANXA2 in the pro-proliferative ERK pathway revealed enhanced activation of ERK (P-ERK) in MDA MB 231 ANXA2 shRNA-3 but not in the MDA MB 231 ANXA2 shRNA-4 and control cells (figures 3.1 and 3.2). These results were not conclusive since the activation was not observed in MDA MB 231 ANXA2 shRNA-4 cells which portray a better knockdown of ANXA2. Further optimization and analysis is necessary for future exploration of this pathway.

Assessment of oxidation of reactive Cys residues in redox sensitive proteins by BIAM labelling upon treatment with EGF indicate that ANXA2 knockdown and control cells show similar redox profiles after EGF treatment (figure 3.4). Treatment of the cells with 50 ng/ml of EGF for the times indicated may just lead to differential mild and localized physiological increases in intracellular ROS levels in the MDA MB 231 ANXA2 knockdown and control cells. In addition, the EGF concentrations used in this study are not high enough to induce supraoptimal ROS levels and the cells were not exposed to exogenous sources of ROS.

Analysis of ROS levels in ANXA2 knockdown versus control MDA-MB-231 cancer cells upon treatment with EGF revealed that ROS levels were significantly higher in ANXA2 knockdown cells compared to control cells after treatment with EGF for 15 minutes (figure 3.5). This implies that ANXA2 plays a role in ROS inactivation upon EGF induced signalling. Therefore, treatment of ANXA2 knockdown MDA-MB-231 cancer cells with EGF leads to an intracellular increase and accumulation of ROS, an effect which is significantly less observed in control cells. This result is in agreement with the results obtained by Madureira *et al.,* in a study which showed that ANXA2 depleted cells accumulate higher concentrations of ROS upon treatment with various concentrations of H_2O_2 in TIME, LCC and MCF7 cancer cells [116]. ANXA2 depleted cells accumulate more H_2O_2 upon treatment with EGF compared to control cells, which leads to enhanced activation of the ROS dependent PI3K pathway as observed in the results of the signalling experiments.

Evaluation of the role of ANXA2 in cell proliferation induced by the EGF/EGFR signalling pathways revealed that ANXA2 knockdown cells have higher rates of proliferation upon EGF treatment compared to control cells (figure 3.6). These results are in line with the aforementioned observations of enhanced activation of the pro-proliferative ROS dependent PI3K pathway in ANXA2 knockdown cells which accumulate more ROS and proliferate at a higher rate compared to control cells upon EGF treatment. Since ANXA2 depleted cells reveal significantly higher levels of ROS as compared to control cells upon treatment with EGF, the ROS so produced could be responsible for the enhanced activation of the PI3K pathway, inhibition of PTEN and increased rate of proliferation observed in the ANXA2 depleted cells compared to control cells. Also, ANXA2 knockdown seems to lead to the

upregulation of PRDX II. Additional research is ongoing in our laboratory to further elaborate/develop and confirm these findings.

5. Conclusions and future perspectives

5.1. Conclusions

Several studies have demonstrated the upregulation of ANXA2 in various types of cancer whereby it has been positively linked with cancer progression, resistance to chemotherapy and radiotherapy, and poor prognosis. However, the function(s) and mechanism of action of ANXA2 in cancer have not been fully described. EGF is a potent inducer of intracellular ROS production for second messenger signalling in normal and cancer cells. The recent elucidation of a redox regulatory function for ANXA2 by our laboratory led us to investigate the role(s) of ANXA2 in EGF induced signalling in cancer cells. The results of this study show that ANXA2 depletion in MDA MB 231 cancer cells leads to enhanced activation of the pro-proliferative PI3K pathway, upregulation of PRDX II, enhanced ROS production and increased rates of proliferation upon EGF treatment compared to control cells. Our results provide more insights into the redox regulatory functions of ANXA2 and help to better understand the ROS dependent mechanisms of cancer development in a bid to provide novel anticancer strategies. Our results demonstrate that ANXA2 plays a redox regulatory role in EGF induced ROS-mediated PI3K/Akt signalling and ANXA2 knockdown cells might be upregulating PRDX II to compensate for the loss of the ANXA2 redox regulatory protein. Additional research work is ongoing to validate and further develop these results.

The results of this work are part of a manuscript in preparation: Castaldo SA, Ajime T, Madureira PA. "Annexin A2 inhibits the PI3K/Akt pathway in cancer cells via the REDOX regulation of PTEN". EMBO. IF: 10.748. My work was also presented in poster format at the special EACR/AACR/SIC conference on anticancer drug action and drug resistance: from cancer biology to the clinic held in Florence, Italy, (20^{th} - 23^{rd} June, 2015) [appendix 1, page 72] and at the oncobiology retreat organised by the CBMR/UAlg on the 20^{th} May 2015 (appendix 3, page 74).

5.2. Future perspectives

In order to validate the above mentioned findings, we will be carrying out the above mentioned procedures in different cancer cell types and with other growth factors (such as platelet derived growth factor [PDGF]) *in vitro* and *in vivo*. Co-immunoprecipitation (Co-IP)

assays will be done to further investigate the interactions between ANXA2 and PTEN in a bid to propose a plausible redox regulatory mechanism for ANXA2 in PI3K signalling.

To further investigate and validate the compensatory upregulation of PRDX II in ANXA2 depleted cells, various cancer cell lines will be established with shRNA mediated knockdown for ANXA2, PRDX II and both. The activation of various signalling pathways upon treatment with H_2O_2, EGF or ROS inducing therapeutics will be investigated to evaluate the physiological outcomes of the knockdowns. Co-IPs will also be done to investigate if ANXA2 and PRDX II might interact or bind to similar target proteins.

Considering the nuclear functions of ANXA2 and the existence of redox regulated transcription factors, we could also investigate the role of ANXA2 in the regulation of the activity of redox sensitive transcription factors.

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Appendix 1: Poster presented at the special EACR/AACR/SIC conference, Florence, Italy, 20th - 23rd June, 2015.

Investigating the role of annexin A2 in epidermal growth factor (EGF) induced signalling

Stéphanie Anais Castaldo[1], Ajime Tom Tanjeko[1,2], Nadine Vasconcelos Conchinha[1], Patricia Alexandra Madureira[1,*]

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Introduction

Over the past decade increasing evidence has shown that the reactive oxygen species (ROS), H_2O_2 is an important second messenger in cell signal transduction, due to its high diffusibility and ability to target reactive cysteine residues in proteins. H_2O_2 is induced by various signalling proteins, including growth factors, cytokines, hormones and neurotransmitters through the activation of NADPH oxidases. Currently, H_2O_2-dependent signalling has been implicated in fundamental processes such as cell proliferation, differentiation, migration and apoptosis.

Cancer cells typically exhibit increased ROS levels compared to normal counterparts that gives them a proliferative advantage and promotes malignant progression. To balance the advantage of low ROS levels (proliferative signalling pathways) versus its damaging effect (as an oxidant at high concentrations), cancer cells induce the cellular antioxidant response.

Our laboratory identified a novel redox regulatory protein, annexin A2 (ANXA2) (Fig. 1) and showed that ANXA2 antioxidant function plays a crucial role in supporting tumour growth and chemoresistance. As a logical continuation to this research we investigated the role played by ANXA2 in the regulation of oncogenic signalling pathways induced by the epidermal growth factor, EGF.

Keywords: annexin A2 (ANXA2); reactive oxygen species (ROS); epidermal growth factor (EGF)

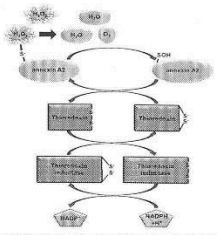

Figure 1. ANXA2 antioxidant function. H_2O_2 oxidizes Cys-8 of ANXA2 resulting in the conversion of H_2O_2 into H_2O and O_2. Oxidized ANXA2 is then reduced by the Trx redox system and can participate in further redox cycles. Thus a single molecule of ANXA2 can degrade several molecules of H_2O_2.

Materials and Methods

Establishment of MDA MB 231 breast cancer cells knockdown for ANXA2 (p36) and controls → Treatment with 15 nM EGF (time-courses) and cell signalling analysis by western blotting → Intracellular ROS levels analysis using the probe 2',7'-dichlorodihydrofluorescein diacetate,DCF-DA → Cell proliferation analysis using CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega®) → Co-immunoprecipitation studies to determine ANXA2 and PTEN binding, antibodies for ANXA2, PTEN or IgG → ANXA2 and PTEN (WT and mutants) co-transfection studies and analysis of PI3K/AKT activation by western blotting

Results

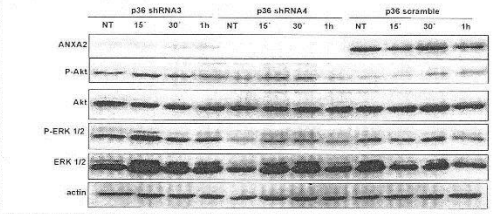

Figure 2. Annexin A2 knockdown cells show enhanced activation of the PI3K/AKT signalling pathway in response to EGF treatment compared to control cells. (A) MDA MB 231 ANXA2 knockdown (KD) cells (p36 shRNA 3 and shRNA4) (left and middle panels) or MDA MB 231 control cells (p36 scramble) (right panel); were treated with 15 nM EGF for the times indicated. Protein lysates were prepared and 20 µg of each cell lysate was subjected to SDS-PAGE, transferred onto nitrocellulose membrane followed by western blot analysis with the antibodies indicated.

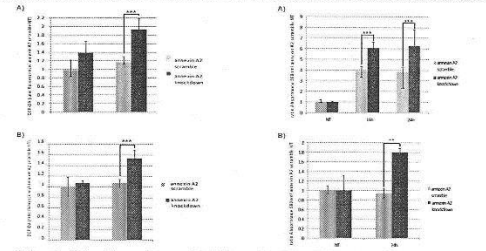

Figure 3. Annexin A2 knockdown cells accumulate higher levels of ROS upon treatment with EGF. MDA MB 231 annexin A2 knockdown or control (scramble) cancer cells were serum starved for 16 hours. Cells were then incubated with 100 µM DCF-DA reagent for 30 minutes followed by mock treatment or treatment with 15 nM EGF for (A) 30 minutes or (B) 1 hour. Fluorescence was measured using a fluorometer plate reader Exc. 492 nm, Em: 530 nm. Data was analysed using the two tailed Student's t test and represented as ± StDev (n=6).

Figure 4. Annexin A2 knockdown cells have a higher proliferative rate upon EGF treatment. A) A549 annexin A2 knockdown or control (scramble) and B) MDA MB 231 cancer cells were serum starved for 16 hours followed by mock treatment or treatment with 15 nM EGF for 16 and 24 hours. Cell proliferation was analysed using the CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega) according to manufacturer's instructions. Abs 560 nm was measured using a plate reader. Data was analysed using the two tailed Student's t test and represented as ± StDev (n=6).

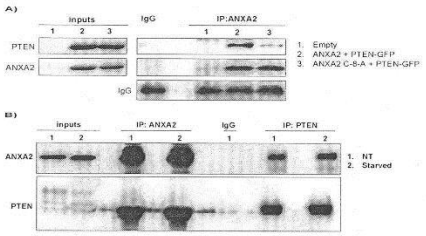

Figure 5. Annexin A2 binds to the PI3K/ AKT signalling pathway inhibitor, PTEN, and annexin A2 Cys-8 reactive cysteine is important for binding. (A) 293T cells were mock transfected (1), co-transfected with ANXA2 wild-type and PTEN-GFP expression vectors (2) or co-transfected with ANXA2 Cys-8-Ala mutant and PTEN-GFP expression vectors for 48 hours. (B) MDA MB 231 cells were either not treated (1. NT) or starved for 16 hours (2.starved). Protein lysates were prepared and 300 µg of each lysate was immunoprecipitated with the antibodies indicated. ANXA2, PTEN or IgG (control), 20 µg of each lysate (inputs) or immunoprecipitates were electrophoresed by SDS-PAGE, transferred onto a nitrocellulose membrane followed by western blotting with the antibodies indicated.

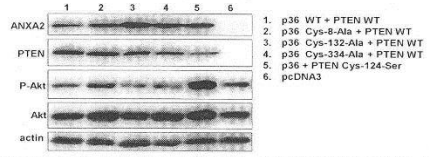

Figure 6. Co-expression of annexin A2 WT but not annexin A2 Cys-8-Ala mutant with PTEN inhibits the PI3K/AKT signalling pathway. (A) 293T cells were transfected with the expression vectors indicated for 48 hours. Protein lysates were prepared and 20 µg of each lysate was electrophoresed by SDS-PAGE, transferred onto a nitrocellulose membrane followed by western blotting with the antibodies indicated.

Summary

Our work showed that annexin A2 knockdown cancer cells have enhanced levels of ROS and over activation of the PI3K/AKT signalling pathway compared to control cells in response to EGF treatment, which resulted in enhanced proliferation of annexin A2 knockdown cells. The PI3K/AKT signalling pathway is negatively regulated by the protein phosphatase and tensin homolog (PTEN). PTEN has two reactive cysteine residues (Cys-124 - Cys-71) within its catalytic domain that can be readily oxidized by ROS, inactivating its phosphatase function. We hypothesized that the enhanced levels of ROS observed in the annexin A2 knockdown cells might lead to PTEN oxidation and to the consequent activation of the PI3K pathway observed in these cells. Furthermore, since annexin A2 contains a reactive Cys-8 residue we investigated if ANXA2 could bind to PTEN and regulate its function. Our co-immunoprecipitation studies showed that annexin A2 binds to PTEN and that Cys-8 residue of annexin A2 is important for its interaction with PTEN. In addition we observed that co-expression of annexin A2 but not annexin A2 Cys-8-Ala mutant with PTEN leads to inhibition of the PI3K/AKT pathway. These results suggest that ANXA2 might be regulating PTEN function via Cys-8 mediated reduction of PTEN reactive cysteines re-establishing in this way PTEN phosphatase function.

Acknowledgements

FCT Fundação para a Ciência e a Tecnologia · Seventh Framework Programme · Marie Curie Actions · Governo de Portugal · Ministério da Educação e Ciência · Erasmus Mundus

Appendix 2: Certificate of attendance for the special EACR/AACR/SIC conference, Florence, Italy, 20th - 23rd June, 2015.

Appendix 3: Poster presented at the spring oncobiology retreat organised by CBMR/UAlg, 20th May 2015

Investigating the role of annexin A2 in epidermal growth factor (EGF) induced signalling

Ajime Tom Tanjeko[1], Stéphanie Anais Castaldo[1], Nadine Vasconcelos Conchinha[1], Patrícia Alexandra Madureira[1,*]

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* This research work has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° PCOFUND-GA-2009-246542 and from the Foundation for Science and Technology of Portugal (FCT).

Introduction

Recently, increasing evidence has demonstrated the importance of reactive oxygen species (ROS), especially H_2O_2, as a second messenger in cell signal transduction, thanks to its high diffusibility and selective targeting of reactive cysteine residues in proteins. H_2O_2 production is induced by various signalling proteins, including growth factors, cytokines, hormones and neurotransmitters. Currently, H_2O_2-dependent signalling has been implicated in fundamental processes such as cell proliferation, differentiation, migration and apoptosis. Cancer cells typically show increased ROS levels compared to normal counterparts, giving them a proliferative advantage which promotes malignant progression. To balance the advantage of high ROS levels versus its damaging effects, cancer cells induce the cellular antioxidant response. Our laboratory identified a novel redox regulatory protein, annexin A2 (ANXA2) and demonstrated its crucial role in enhancing tumour growth and chemoresistance. As a logical continuation to this research we are investigating the role of ANXA2 in the regulation of oncogenic signalling pathways induced by the epidermal growth factor (EGF).

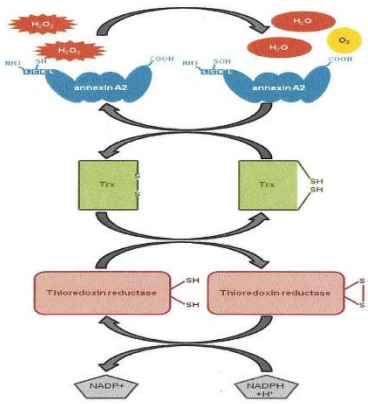

Figure 1. ANXA2 antioxidant function.
H2O2 oxidizes Cys-8 of ANXA2 resulting in the conversion of H2O2 into H2O and O2. Oxidized ANXA2 is then reduced by the Trx redox system and can participate in further redox cycles. Thus a single molecule of ANXA2 can degrade several molecules of H2O2.

Materials and Methods

Cancer cell lines (A549 lung cancer, MDA breast cancer and HT1080 fibrosarcoma) were established with knockdown for ANXA2 and respective control cells (scramble) and the activation of signalling pathways upon treatment of these cells with 50 ng/ml EGF at different time points (time courses) was investigated by western blotting. We analysed the levels of intracellular ROS in the ANXA2 depleted versus control cancer cells upon treatment with EGF, using the probe 2',7' dichlorodihydrofluorescein diacetate (DCF-DA). We studied the proliferation of ANXA2 depleted versus control cancer cells upon treatment with EGF using CellTiter 96® assay (Promega), according to the manufacturer's instructions.

Results

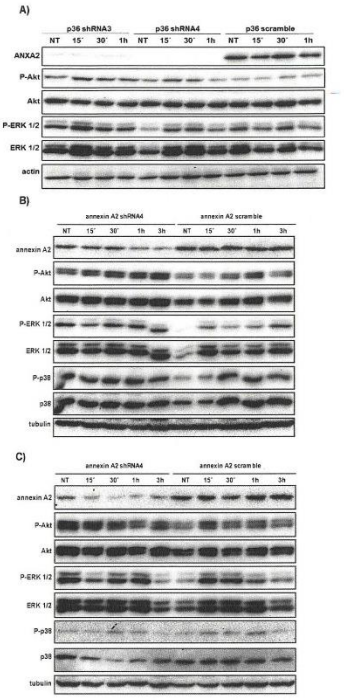

Figure 2. Annexin A2 knockdown cells show enhanced activation of the PI3K signalling pathway in response to EGF treatment compared to control cells. (A) MDA MB 231 knockdown cells (annexin A2 shRNA3) (left panel), MDA MB 231 annexin A2 shRNA4 (middle panel) or control cells (annexin A2 scramble) (right panel); (B) HT1080 KD cells (annexin A2 shRNA4) (left panel) or MDA MB 231 control cells (annexin A2 scramble) (right panel); (C) A549 knockdown cells (annexin A2 shRNA4) (left panel) or A549 control cells (annexin A2 scramble) (right panel) were treated with 50 ng/ml EGF for the indicated times followed by SDS PAGE, western blots and immunochemiluminescent identification with the above mentioned monoclonal antibodies.

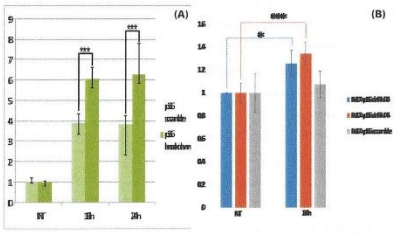

Figure 3. Annexin A2 knockdown cells have a higher proliferative rate upon EGF treatment. A549 annexin A2 (p36) knockdown or control (scramble) and MDA MB 231 p36 knockdown or scramble cancer cells were serum starved for 16 hours followed by mock treatment or treatment with 50 ng/ml EGF for 16 and 24 hours (A549) **(A)** and 24 hours (MDA MB 231) **(B)**. Absorbance (570nm) was measured after a tetrazolium reduction cell proliferation assay.

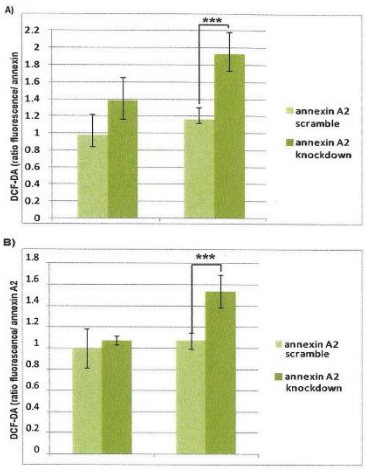

Figure 4. Annexin A2 knockdown cells accumulate higher levels of ROS upon treatment with EGF. A549 annexin A2 knockdown or control (scramble) cancer cells were serum starved for 16 hours followed by 30 minutes incubation with 50 µM DCF-DA reagent and mock treatment or treatment with 50ng/ml EGF for (A) 30 minutes or (B) 1 hour. Fluorescence was measured (Exc. 492 nm, Em: 524 nm).

Summary

Our results showed that annexin A2 knockdown cells have enhanced levels of ROS and over activation of the PI3K signalling pathway (increased phosphorylation of Akt) compared to control cells in response to EGF treatment, which resulted in enhanced proliferation of annexin A2 knockdown cells. The PI3K pathway is negatively regulated by the protein phosphatase and tensin homolog (PTEN) which has two cysteine residues (Cys-124 ; Cys-71) within its catalytic domain that can be readily oxydized by ROS, inactivating its phosphatase function. The enhanced levels of ROS observed in the annexin A2 knockdown cells might lead to PTEN oxidation and to the consequent activation of the PI3K pathway observed in these cells. Additional research work is ongoing to investigate this hypothesis.

Acknowledgements

Appendix 4: Composition of running and stacking gels for SDS-PAGE

Running gels			**Stacking gel**	
(acrylamide:bisacrilamide) %	**10%**	**12%**	(acrylamide:bisacrilamide) %	**5%**
1M Tris pH 8.8	3 ml	3 ml	1M Tris pH 6.5	312.5 µl
40 % acrylamide:bisacrylamide	2 ml	2.4 ml	40 % acrylamide:bisacrylamide	312.5 µl
Distilled H_2O	2.9 ml	2.5 ml	Distilled H_2O	2.6 ml
10 % SDS	80 µl	80 µl	10 % SDS	25 µl
25 % APS	32 µl	32 µl	25 % APS	12.5 µl
TEMED	12 µl	12 µl	TEMED	7.5 µl

Appendix 5: Buffers for SDS-PAGE

10 X running buffer stock

- 250 mM Tris base
- 2.5 M glycine
- 1 % SDS

(Gels were run using 1 X running buffer.)

10 X Transfer Buffer stock (without methanol)

- 250 mM Tris base
- 2 M glycine

(Transfers were done using 1 X transfer buffer with 20% of methanol per unit volume.)

4 X protein loading buffer

- 8 % SDS
- 120 mM Tris pH 6.8
- 20 % glycerol
- 0.02 % bromophenol blue
- 20 % β-mercaptoethanol

20 X TBS buffer

- 400 mM Tris pH7.5
- 2.4 M NaCl

(Working solutions of 1 X TBS-T (1 X TBS + 0.1% tween 20) were used to wash nitrocellulose membranes.)

Appendix 6: Composition of the enhanced chemiluminescence (ECL) solution

Solution 1:

- 20 ml 1 M Tris, pH 8.5 (100 mM Tris pH 8.5)
- 2 ml luminol (2.5 mM luminol)
- 889 μl p-coumaric acid (360 uM p-coumaric acid)
- 178 ml H_2O

Solution 2:

- 20 ml 1 M Tris, pH 8.5 (100 mM Tris pH 8.5)
- 180 ml H_2O
- 123 μl 30% H_2O_2 (0.0185 % H_2O_2)

(Working solutions of the ECL were prepared as 1 : 1 mix of solutions 1 and 2 in the absence of light.)

stock solutions:

- 250 mM luminol (3-aminophtalhydrazid) in DMSO
- 90 mM p-coumaric acid in DMSO
- keep solutions at 4ºC

Chemicals:

- p-Coumaric acid - Sigma C-9008
- 5-Amino-2,3-dihydro-1,4-phthalazidedione (3-aminophtalhydrazid) = Luminol - Sigma A-8511
- Hydrogen peroxide - Sigma H-1009