Understanding the Apoptotic Signaling Pathways In Breast Cancer Using Microarrays, Proteomics and Bioinformatics

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Abstract

Breast cancer is one of the most common causes of cancer death to women worldwide. In this study A431 cells, derived from epidermoid carcinoma, are used as a model to study breast cancer. This cell line over-expresses the Epidermal Growth Factor Receptor (EGFR/HER1) and when treated with a high dose of EGF will undergo apoptosis via the activation of EGFR/HER1 signaling. However, little work has been conducted to identify the underlying molecular mechanisms. The limited available data implicates components of the interferon response pathway as mediators of the apoptotic signal in cancer cells. The genetic network through which EGFR/HER1 can induce apoptosis is not known at the present time. With understanding of the genetic regulatory hierarchy and the molecular mechanisms linking EGFR/HER1 signaling and apoptosis, better drug targets that might regulate apoptosis in cancers that over express HERs can be identified. This thesis focuses on the hypothesis that an apoptosis specific signaling cascade can be triggered by HERs in A431cells. Activation of HER receptors has led us to identify downstream components by global analyses of gene expression and associated regulatory miRNAs and protein levels using microarray and proteomics platforms. A high dose of EGF leads to the induction of apoptosis in A431 cells by activating a number of pathways, which are known to promote apoptosis. These include the STAT pathway and downstream components including cytokines and suppressors of cytokine signalling, cleavage of serpinb1 into L-DNAaseII and down regulation of mutant TP53, which may perturb the cytoskeleton and cell adhesion proteins. Furthermore, our data showed that gene expression and proteomic data were quite different, with very little overlap in terms of transcripts and proteins. Therefore, we considered that post-transcriptional regulation might be crucial. MicroRNAs are known to be important post-transcriptional regulators; as a result, we sought to identify potential regulatory miRNAs with respect to induction of cell death by EGF. We identified novel interaction regulatory networks based on the crosstalk between miRNAs and mRNA/protein that resulted in the induction of apoptosis in A431 cells. We also identified a number of miRNAs that may play an important role in the regulation of apoptosis in A431 cells after EGF treatment. We have used bioinformatics databases and *in silico* analysis tools to create a comprehensive catalogue of genes/proteins that may induce apoptosis in A431 cell after EGF treatment.

We have shown that using various complimentary platforms including gene expression, miRNA expression, proteomics, and network prediction to analyse the induction of apoptosis in treated A431 cells, we have achieved a greater understanding of the molecular mechanisms at work. This in turn may provide strategies for combined therapies and/or predict novel potential mechanisms/targets for chemotherapy aimed at inducing cell death in tumor cells.

Declaration

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Combined gene expression and proteomic analysis of EGF induced apoptosis in A431 cells suggests multiple pathways trigger

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CHAPTER ONE

1 Introduction

Breast cancer is on the most common diseases affecting women around the world. The contribution of genetic factors in breast cancer including breast cancer gene 1 (*BRCA1*) and breast cancer gene 2 (*BRCA2*) is estimated to be about 5 to 10% [1]. The over expression of epidermal growth factor receptor EGFR (*HER1*) and *HER2* genes may also cause breast cancer [2, 3]. HER1 expression is detected in about 40% of breast cancer [2] while amplification or the over expression of the *HER2* gene occurs in up to 25 to 30% of patients with breast cancer [4-7]. Over expression of HER1 and HER2, which are preferred interacting partners for dimerization in breast cancer, is associated with a poorer clinical prognosis and predicts a bad response to endocrine therapy [8]. In this thesis, A431 cells, derived from epidermoid carcinoma, are used as a model to study breast cancer. There are two reasons of chosen A431(derived from epidermoid carcinoma) cells, they overexpress EGFR (HER1) and represent a well-established model system routinely used in RTK activation and network modeling studies [9]. HER1 is overexpressed in a number of human malignancies including cancer of breast. Moreover, increased HER1 expression correlates with a poorer clinical outcome for patients with breast cancer [10]. The A431 cell line over expresses HER1 and when treated with a high dose of epidermal growth factor (EGF) will undergo apoptosis via the activation of HER1 signaling [11-13]. A431 cells were utilized as a test cell line because of their extensive previous use for the analysis of HER1 and other HER receptors. However, little work has been done to identify the underlying molecular mechanisms. The limited available data implicates components of the interferon response pathway as mediators of the apoptotic signal in cancer cells [13, 14]. At the present time, the genetic network through which HER1 can induce apoptosis is not known. Without a detailed understanding of the genetic regulatory hierarchy and the molecular mechanisms linking HER1 signaling and apoptosis, better drug targets that would regulate apoptosis in cancers that over-express HERs cannot be identified. This thesis focuses on the hypothesis that apoptosis specific signaling cascade can be triggered by HERs in transformed cells (A431cells). To address this hypothesis, a number of assays including XTT and Caspase-3/CPP32 colorimetric assays have been used determine the time points for RNA and protein extraction; and platforms including Microarray and Proteomics platforms have been utilized to identify gene expression

and protein profile respectively. Therefore, using these techniques assist in understanding the apoptosis-signaling cascade and identifying downstream components (immediate early response and delayed response genes) of this cascade through activation of HERs. This is crucial since downstream genes are more likely to be useful targets for chemotherapy.

1.2 Literature Review

1.2.1 Mammary Development And Function

The mammary gland (breast) differentiates mammals from all other animals with its distinctive anatomical structure that secretes milk for the nourishment of the newborn. Over 300 million years ago, mammary glands, which are epidermal outgrowth, were evolved; most likely from apocrine sweat glands [15]. The mammary gland is a distinctive dependence on hormonal signals for terminal differentiation, which is achieved only after pregnancy. Intensive efforts have been conducted during the past 100 years ago to understand the endocrine control of mammopoiesis and lactogenesis. Classical research on endocrine ablated animals indicated that ovarian steroids and pituitary peptide hormones are compulsory and adequate for breast development and lactation [16, 17]. It was discovered in 1900 that the ovary has the ability to control mammary growth and when ovaries are transplanted, the castration atrophy of mammary glands is prevented [16, 17]. A number of studies have also demonstrated that mammary regression can be caused by ovariectomy [16, 17]. The identification of the genetic components of the mammary gland development has been achieved by deleting genes from the mouse genome. Experimental manipulations of tissues from wild-type and knockout mice have been used to delete these genes [16, 17]. When genes have been removed from the mouse genome (Table 1), impaired mammary gland development has resulted. This has allowed the recognition of the fundamental genetic framework and signaling networks of the developing tissue.

Two discrete developmental concepts have been unfolded. Systemic endocrine hormones activated distinct signaling networks that induce mammopoiesis and some of these signaling transmitted through interactions between the epithelium and the stroma [16]. The role of systemic hormones and the influence of the stroma on mammary epithelial cells have been

distinguished. Nevertheless, the individual steps in the pathways of the translation of hormonal signals into morphogenetic and developmental events can be analyzed only now through the availability of knockout mice model [16]. The hormonal status of the animal is used to define the functional development of the mammary gland through distinct stages. The establishment of mammary anlage occurs during fetal development, while ductal elongation and branching happens after the beginning of puberty [16]. During pregnancy, alveolar proliferation occurs while functional differentiation is accomplished with parturition and lactation [16, 17]. The regulation of interaction between epithelial-stromal by hormones is of major importance given that hormones initiate diverse phases of mammary gland development and influence the progression of breast cancer [18]. The mammary gland has emerged as a rich developmental model because it depends on epithelial–stromal interactions. The use of reverse genetics leads to the generation of more than ten mutants that affect mammary development. According to Vainio and Mueller, analyses of these mutants lead to the emergence of a model for mammary development. Various hormones such as estrogens and progesterone are implicated in epithelial-stromal interaction control mammary development. Growth factors secreted locally become candidates to operate as mediators of the stromal PR-dependent signal. CSF-1, EGF and members of the TGFb family may possibly be involved [16, 18]. It has been proven that the stromal EGF receptor (EGFR) is required for ductal development. Therefore, EGF is one of the main candidates to transmit systemic signals between stroma and epithelium [16].

Table 1: Knockout mice and natural mutants that exhibit a mammary gland phenotype [16].

The making of a mammary gland

Hormones, growth regulators, and receptors	Transcription factors	Cell cycle	Others
Prolactin (PRL) (1)	Progesterone R (*PR*) (6)	cyclin D1 (12)	LAR (13)
Prolactin R (PRLR) (2)	Estrogen R (*ER*) (7)		
Oxytocin (3)	Stat5a (8)		
InhibinβB (4)	A-myb (9)		
CSF-1 (5)	mf3 (10)		
	C/EBPβ (11)		

1.2.1.1 The Human Epidermal Growth Factor Receptors (EGFRs) And The Role Of Their Signaling During Mammary Development And Neoplasia

The human epidermal growth factor receptor (EGFR) or the HER family of proteins consists of HER1, HER2, HER3 and HER4 which are type 1 tyrosine kinase growth factor receptors [19, 20]. EGFR has a similar sequence to the v-erb-B oncogene [19]. The previous types of EGFR receptors are known as (v-erb-B / HER1, v-erb-B2/ HER2, v-erb-B3/HER3, v-erb-B4/HER4) [21, 22]. HER receptors are expressed in a diversity of tissues of epithelial, mesenchymal and neuronal origin. They have essential roles in proliferation, development and differentiation [23]. Each receptor consists of an extracellular domain, transmembrane domain and an intracellular protein tyrosine kinase domain. Receptor dimerization is an essential requirement for HER function and for the signalling activity of HER receptors. Dimerization can be classified into two classes; heterodimerization and homodimerization. Heterodimerization occurs between two different HER receptors while homodimerization occurs between two molecules of the same receptor [24]. The ability of HER receptor dimers to stimulate various signal transduction pathways has been investigated, and relevant findings are presented below. The phenotype of knock-out mice showed that a heterodimer can attain novel signaling properties that are not the

sum of the activity of individual receptor dimers. For instance, the neuregulin (NRG-1) ligand activates HER4 dimers or HER 2/ HER 4 heterodimers. Each of these HER dimers induce intracellular MAP kinase activity. However, only the HER 2/ HER 4 heterodimers stimulate activation of Stat5, a member of the signal transducer and activator of transcription (Stat) family [23]. HER3 and HER4 acquire direct binding sites for the p85 subunit of PI3K which leads to the activation of PI3K and its downstream signaling components [25] .

HER3 lacks innate kinase function, however, HER3 heterodimerizes with other HER receptors. HER3 appears to be the preferred dimerization partner when signaling occurs through the PI3K pathway[1] and therefore, HER3 is emerging as a key target for inhibition of HER signaling. The HER2– HER3 dimer is important for HER2-mediated signaling in tumours containing amplifications of *HER2*. HER2– HER3 is believed to be the most active HER signalling dimer. Also, HER3 plays an important role in human tumorigenesis [24].

HER family members are often over-expressed in many forms of cancer. For example, the deregulation of HER receptors expression, in particular HER 1 and HER 2, has been implicated in the malignancy of numerous types of human tumors, including breast cancer [23]. The over expression of HER 1 and HER 2 is associated with a poorer clinical prognosis and predicts a bad response to endocrine therapy [8]. HER1 and HER 2 interact with and activate PI3K through the adaptor proteins GRB2 (growth factor receptor-bound 2) and GAB1 (GRB2-associated binding protein 1) [26].

HER1 is a single chain transmembrane polypeptide protein that consists of three domains. These domains are the extracellular, transmembrane and intracellular tyrosine kinase domains. The extracellular domain permits ligands to bind to it and then activate the receptor. The transmembrane domain engages in dimerization interaction between receptors. The intracellular tyrosine kinase domain has the ability to phosphorylate tyrosine residues on substrate proteins. This domain has a carboxyl-terminal tail containing tyrosine autophosphorylation sites [27]. These sites bond to proteins that contain Src homology 2 and phosphotyrosine binding domains (PTB) [27, 28]. Therefore, these proteins activate a large number of signal transduction

molecules such as protein kinase B (PKB or AKT) tyrosine kinase Src, c-Jun Kinase, stress-activated protein kinase and single transducer and activators of transcription (STATs) [27].

HER receptors play an important role during the development of the mammary gland. Several experiments reveal the role of *HER1* in mammary ductal development. It has been proven that by using expression of a dominant-negative (DN) HER-1 experiment or using a reconstitution experiment utilizing *HER1* knockout (-/-) neonatal mammary gland [28]. It has been found that a defect in expressing of *HER* receptors leads to a defect in mammary ductal growth. Using expression of a dominant-negative (DN) HER1 experiment indicates that defects in expressing of *HER2* and 4 receptors do not affect the normal ductal growth but lead to alterations in lobular alveolar development and milk protein production. The previously mentioned experiment also shows that *HER3* is required for the development of normal lobular alveolar [28].

Abnormal HER signaling leads to neoplastic progression in mammary glands. Several transgenic mouse models have been used to study the effect of unregulated HER signaling in the mammary gland. For instance, the over expression of *TGFα*, which is controlled by mammary tumor virus (MMTV LTR) promoter, leads to the exhibition of anomalous development of mammary gland including precocious emergence of alveoli and impaired postlactational involution. This leads to the persistence of epithelial structures that are termed hyperplastic alveolar nodules. Focal mammary tumors that have a high effectiveness and quite long latency also can be developed [29]. Furthermore, HER1 and their ligands are capable of inducing in vivo transformation. For example, mammary tumor virus MMTV or β-lactoglobulin (BLG) promoter in the mammary glands of virgin transgenic mice controls the expression of *HER1*. This expression develops mammary hyperplasias that progresses to dysplasias and tubular adenocarcinomas in lactating animals [28]. However, the over expression of *HER1* using certain promoters may lead to the raising of proliferation levels, but does not form a tumor in different tissues such as esophageal keratinocytes glial cells and urothelium. It has been found that the overexpression of *HER1* alone will not lead to in vivo transformation unless other factors also contribute. These include factors such as inactivation of tumor suppressor genes and activation of proto-oncogene, a normal gene that has the potential to become an oncogene [29].

1.2.1.2 The Role Of Hedgehog, Wnt And Notch Signaling During Mammary Development And Neoplasia

Several signaling pathways including Hedgehog, Wnt and Notch signaling play key roles during mammary gland development and neoplasia. Several studies have been performed on transgenic mice that prove the role of Hedgehog (Hh) signaling in the mammary gland [30-32]. For instance, heterozygous disruption of Ptch1, which is the receptor of this signaling in virgin mice, is responsible for abnormalities in mammary ductal structures as well as hyperplasias and dysplasias. Other studies indicate that the MMTV promoter is used in mice to control human Smoothened (Smo) [31, 32]. The activation of human Smo leads to increased proliferation, altered differentiation and developed ductal dysplasias in mammary ductal cells. The association between aberrant hedgehog signaling and breast cancer has been identified using immunohistochemistry for Hh signaling components. The results show that normal duct cells express less Sonic hedgehog (Shh), Ptch and Gli activator 1 (Gli1) than tumours. However, the Hh mechanism activation in breast cancer is still unclear [31]. Wnt signaling plays an important role in embryonic tissue patterns and involves in cell migration, maintenance of stem cells and cells adhesion according to Nelson and Nusse [33]. Canonical Wnt signaling has been implicated in breast carcinoma and many other tumours. The Canonical Pathway is involved in the stabilization and translocation of β-catenin to nucleus that is implicated in a number of human malignancies [31]. Notch signaling also plays a role during mammary development and neoplasia. The abnormality of Notch Signaling causes the development of malignant phenotype in human and mouse. This signaling is reported to be important in epithelial carcinoma including breast cancer, colon and lung. Over-activating of the Notch 4 gene using MMTV promoter is responsible for arresting mammary gland development with deprived ductal branching and finally poorly differentiated adenocarcinomas will develop in transgenic mice. Moreover, the overexpression of Notch1 and Notch2 leads to the elevation of tumor grade and is associated with poor prognosis; and constantly plays a role in human breast carcinoma [31]. According to Callahan and Egan, Notch Signaling also has the ability to upregulate the HER-2 gene [34].

1.2.2 Progression Of Breast Cancer

1.2.2.1 Causes Of Breast Cancer

Breast cancer is one of the most common cancers among women. The possibility of having breast cancer is high if there is a family history of breast cancer, especially if a close relative such as a mother, sister or daughter has been diagnosed with breast cancer at a premenopausal age [3]. There were an estimated 421 000 new cases of breast cancer diagnosed in Europe and about 1.4 million worldwide in 2008. The occurrence of breast cancer around the world is predicted to increase to 2.3 million by 2030, although mortality rates in Western countries are declining due to earlier detection and effective adjuvant treatment [35]. The contribution of genetic factors in breast cancer is estimated to be about 5% of all cases but roughly 25% of cases are diagnosed before age 30 [1]. Breast cancer has been classified into two types including early-onset and late-onset based on an inflection in the age specific incidence curve around age 50. BRCA1 gene mutation is found in around 45% of families with high breast cancer occurrence and 80% of families with incidence of early onset breast cancer and ovarian cancer [1]. Moreover, BRCA2 roughly accounts for the equivalent percentage as BRCA1. However, BRCA2 may not influence ovarian cancer risk [1, 36].

The approximate percentage of women born with a mutation BRCA1 and BRCA2 is 5 to 10 % [37]. In 1990, the BRCA1 gene was isolated and mapped to chromosome arm 17q21 [1, 36, 38-40] while, the BRCA 2 has been recently mapped to chromosome arm 13q12-13 [1, 36, 41]. The BRCA1 gene encodes protein that is a negative regulator of tumour growth. The hypothesis that BRCA1 is a tumour suppresser gene may be accurate because its protein has been observed in normal breast and ovarian epithelium tissue and any absense or reduction of this protein has been obtained in some breast and ovarian tumours [1].

The human BRCA1 gene encodes a protein of 1863 amino acids (Figure 1 A) [1, 42]. This gene consists of 22 coding axons spread over approximately 100 kb of genomic DNA [1]. The BRCA1, which has an impact on DNA repair, transcriptional regulation, cell-cycle progression and meiotic sex chromosome inactivation, is an E3 ubiquitin ligase. Green and Rheinwald note that BRCA1 is capable of repairing the DNA and any defect in this gene leads to defect in DNA-repair processes. This gene has an impact on transcriptional regulation by association with the RNA polymerase II holoenzyme complex, but is not essential for transcription. The BRCA1 is also required in the cell cycle [42]. BRCA1 functions as a checkpoint mediator. The BRCA1 is

required for the S-phase and G2/M checkpoint arrest in response to DNA damage. Moreover, BRCA1 is also able to form multiple spindle poles in a signal cell [42].

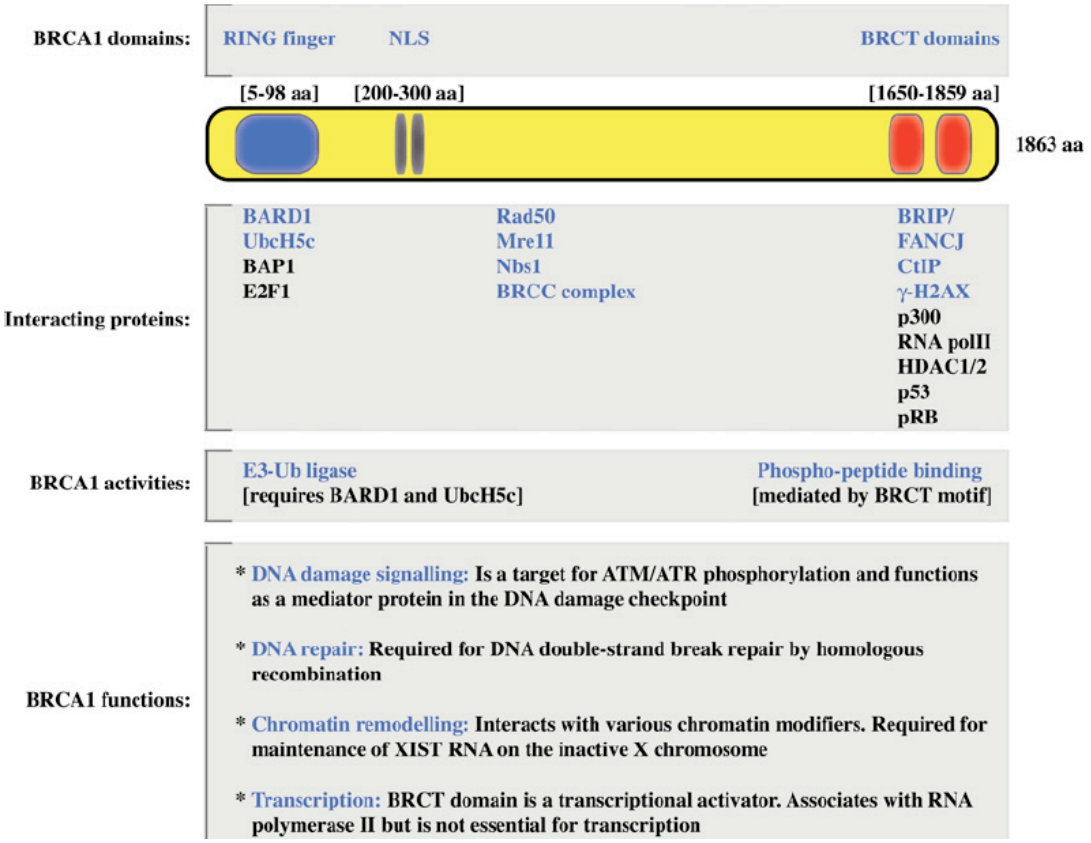

Figure 1 A: BRCA1 domains, interacting proteins, activities and functions. Schematic diagram of the BRCA1 protein depicting the position of the RING, nuclear localization signal (NLS) and BRCT domains [42].

Wooster et al's experiments show that BRCA2 leads to a high risk of early-onset breast cancer in females and a lower risk of ovarian cancer compared to BRCA1. Moreover, there is strong evidence indicating that males with breast cancer have a mutation in the BRCA2 gene. In contrast, it has been observed that there is no male breast cancer in families showing strong evidence of linkage to BRCA1. Therefore, men who are carrying BRCA2 mutations have a higher risk of breast cancer than men who carry BRCA1 mutations. Wooster et al's show that BRCA1 and BRCA2 genes do not account for all breast cancer caused by high-risk susceptibility genes [36]. The human BRCA2 gene encodes a 3418-amino-acid protein considered to be one of the largest polypeptides in the human proteome and BRCA2 function can be seen in figure 1 B [42].

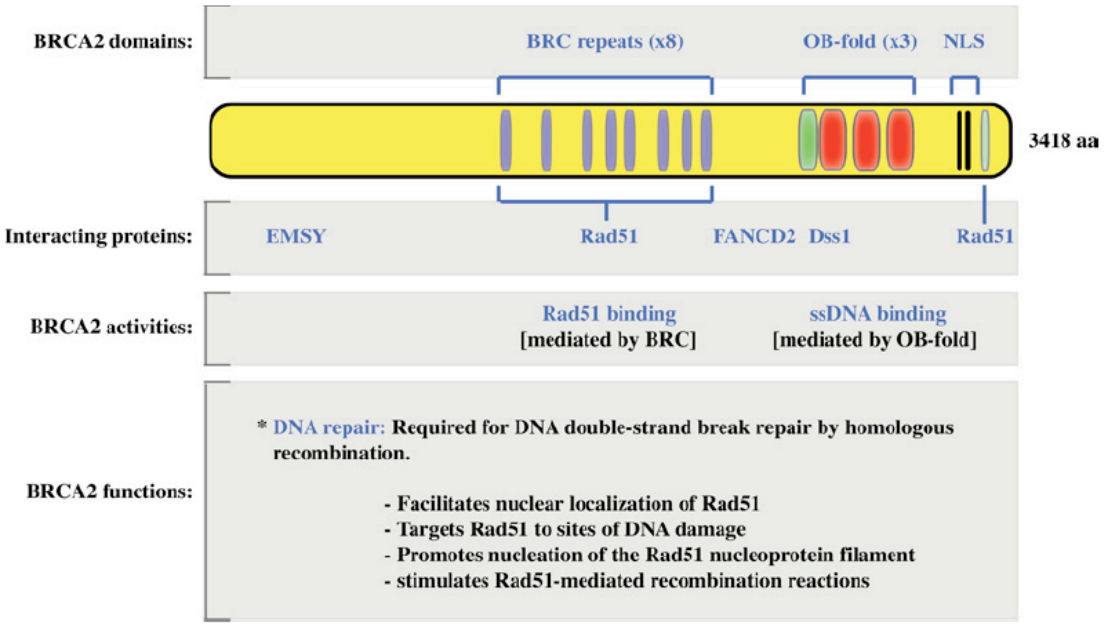

Figure 1 B: BRCA2 includes domains, interacting proteins, activities and functions. Schematic diagram of the BRCA2 protein depicting the position of the eight BRC motifs (blue), the DNA-binding region [helical domain (yellow)], OB-folds (red) and the single Rad51-binding site (green) at the extreme C-terminus, regulated by Cdk phosphorylation [42].

The remaining susceptibility to early-onset breast cancer may attribute to the unmapped gene for familial cancer and odd germline mutation in genes such as TP53 gene [1]. This gene encodes P53 protein that is considered to be a tumor suppressing protein [1, 43, 44]. Moreover, Heterozygote carriers can be at higher risk of breast cancer if these carriers have defective forms of the gene predisposing them to ataxia-telangiectasia mutations (ATM). Mutation in p53 and ATM may contribute to a small minority of breast cancer families that are unlinked to BRCA1 [1, 36, 44, 45]. Late-onset breast cancer is frequently ancestral in origin, but the risks in relatives who have late-onset breast cancer are less than those for early-onset breast cancer. The percentage of cases related to genetics is still unknown [1]. In the pathogenesis and progression of breast cancer, some of the key genes have been identified such as loss of heterozygosity on chromosomal loci 13q,9p and 16q which involve *rb* (the retinoblastoma gene), *CDKN2* (encoding the p16 protein), and *CDH1* (encoding the E-cadherin protein). The amplification of

HER2, *c-myc*, *cyclin D1* and insulin-like growth factor genes are common types of genetic alteration [3].

1.2.2.2 Molecular Causes Of Malignant Or Invasive Breast Cancer

Metastasis is an evolutionary process where diverse interactions between cancer cells and their microenvironment yield variations. As a result, these cells go beyond their programmed behavior. Metastatic cancer cells have the ability to populate and grow in new tissue environments and then may lead to organ dysfunction and death [46]. Metastases tumors can be initiated in diverse sites such as the breast or lung. These tumors are thought to behave like the origin tissue with characteristic patterns and kinetics of spread, and discrete profiles of chemo-sensitivity. Therefore, they are treated in a different way [46]. The percentage of women diagnosed with metastatic breast cancer is about 4-6% [47]. The median survival from diagnosis of breast metastatic disease is 2–3 years, but it might be increase to be 10-15 years [35]. Several genes such as epiregulin **(***EREG*), Cyclooxygenase-2 (*COX-2*) and matrix metalloproteinase1 (*MMP-1*) are involved in metastasis progression [48]. These genes assist breast cancer cells to spread to the lung and cooperate in remodeling the vasculature in positions of mammary tumors and lung metastasis. The preventing and treating of metastasis cancer can be achieved if the molecular players and processes, which are involved in metastasis, understood. This may lead to effective targeted approaches against this type of cancer.

1.2.2.3 Basic Concepts Of Metastasis

1.2.2.3.1 Origins Of Cellular Heterogeneity

Primary tumours are comprised of heterogeneous populations of cells that are genetically altered. This alteration permits these cells to permeate physical boundaries, distribute, and colonize a distant organ. A series of these individual processes result in metastasis. In animal models, the percentage of cancer cells that enter the circulation and develop into metastases is approximately 0.01% or less. The occurrence of alterations that is required to obtain metastatic capacity can be increased by the instability of native genomic of cancer cells. For instance, losing the tumour-

suppressor protein P53, which responds to damage DNA and then leads to induce apoptosis or arrest cell growth, is responsible for 50 % of cancers. Hence, cells will be accumulated with DNA damage [46].

1.2.2.3.2 Selective Pressures Of The Tumor Microenvironment

Tissues from various organs have their own physical structure and functional anatomy full with a vascular supply, compartmental boundaries, and a distinctive extracellular milieu of nutrients and stroma. Cancer cells will be exposed to environmental stresses such as a depletion of oxygen and nutrients, reactive oxygen species, a low pH, if they evade this organization. Cancer cells that survive these stresses will be able to form aggressive colony [49]. For instance, Acute cellular hypoxia stabilizes and activates transcription factor, hypoxia-inducible factor (HIF) which in turn regulates tarnscrption of many genes involve in metabolic programming, invasion and metastasis [50]. The activation of HIF via hypoxia in primary breast cancer also leads to increased expression of chemokine receptor 4 (CXCR4), which increases breast cancer cells metastatic ability [51].

1.2.2.3.3 Cancer Stem Cells And Metastasis

The research shows that self-renewing cancer stem cells initiate and maintain different types of cancers. Cancer stem cells may be capable of resisting apoptosis and DNA damage that caused by drugs [52]. The establishment of primary and metastatic tumours would be supported by such attributes. It is thought that the chemokine stromal-cell–derived factor 1 (SDF-1)–CXCR4 axis has the ability to function in support of stem cells or precursor cells and cancer cells [53]. The description of a premetastatic has been obtained from an animal model. When bone marrow derived progenitor cells home to precise distant sites before the formation of a metastasis, this is called a premetastatic niche [54]. Stem cells have the ability to avoid destruction and survive in distant sites. This illustrates why micrometastases can stay inactive even when the primary tumour is removed. The tumour may reappear years later.

1.2.2.4 The Environment Of The Primary Tumour

1.2.2.4.1 Invasion And Epithelial To Mesenchymal Transition

Intercellular adhesion is reduced in many primary tumours that have invasive properties. The reduction of intercellular adhesion frequently results from losing E-cadherin that is a direct mediator of cell–cell adhesive interactions [55]. Several studies show that the abnormal expression of E-cadherin leads to the development of metastases in breast cancer and other cancers [56, 57]. E-cadherin has the ability to mediate calcium-dependent intercellular adhesion and is involved in epithelial cell-to cell adhesion. E-cadherin is also an important regulation of morphogenesis and as a tumour suppressor gene [56]. The loss of both the estrogen receptor (ER) and E-cadherin genes leads to disease progression in invasive carcinomas [46, 56] and metastasis of the breast [46]. These genes also have been methylated during breast cancer progression. However, there is no association between E-cadherin expression and the ER, PR or HER2 (HER-2/neu) status [56]. A variety of mechanisms such as mutations result in an inactive protein, gene silencing by promoter methylation, or down-regulation stimulated by growth factor receptors including EGFR, fibroblast growth factor receptor [FGFR], insulin-like growth factor I [IGF-I] receptor, and MET or SRC family kinases may lead to the loss of E-cadherin [46, 58]. Loss of E-cadherin function in essential, but is not sufficient for epithelial-to-mesenchymal transition, which epithelial cells change to a mesenchymal progenitor-cell phenotype, enabling detachment and reorganization of epithelial-cell sheets during invasion and metastasis [59].

1.2.2.4.2 Motility And Extracellular-Matrix Remodeling

The crucial role of the Extracellular-Matrix (ECM) is to regulate cellular proliferation and differentiation [3]. ECM serves as a support structure or scaffold for cells to attach and move on. This means cells can be attached together by their receptors called integrins and to ECM components such as fibronectin, collagen, and laminin [3, 56]. Integrins are capable of mediating attachment to the actin cytoskeleton by interacting with a cytoplasmic complex that consists of focal adhesion kinases and SRC family kinases [56]. ECM signals trough calcium-dependent guanosine triphosphatases (GTPases) cause cytoskeletal changes that form filopodia (individual cytoplasmic extensions). Filopodia coalesce into larger lamellipodia, structures that are essential in migratory movement [58]. ECM has the ability to create an environment in which tumour cells proliferate and provide a limited barrier for tumour growth. Basement membranes are a specialized type of ECM that can be invaded by tumour cells. Tumour cells are capable of

degrading several components of the matrix by releasing various enzymes such as protease, glycosidase and collagenases. This will permit these cells to invade through tissue barriers, lymph channel walls and blood vessel switch may lead to the releasing of polypeptide factors which can alter protoglycans type created by host mesenchymal cells. For instance, a number of growth factors including hepatocyte growth factor (HGF), insulin-like growth factors (IGF) 1 and 2, EGF, TGF-α, TGF-β, interleukin-6, fibroblast growth factors (FGF)-2 and 10 and metalloproteases-1 (MMP-1) and MMP-7 can be released when fibroblast is activated in the tumour stroma. These factors have the ability to stimulate cell proliferation, inhibition apoptosis and alter cell differentiation [3].

1.2.2.4.3 Stromal Interactions

Cancer cells can permeate the structural boundaries of the primary tumour and can also co-opt local and bone marrow derived stromal cells that respond to their advantage. Tumour-derived colony stimulating factor 1(CSF-1) stimulates tumour-associated macrophages to proliferate and produce growth factors such as fibroblast growth factor (FGF), EGFR ligands, and platelet-derived growth factor [PDG*F*]) and proteases such as MMP*s* and cathepsins in the basement-membrane invasion of mouse tumours [60, 61]. Furthermore, a particular type of carcinoma-associated mesenchymal cell, which is the myofibroblast, can be activated by tumour-associated macrophages, and then the myofibroblast will secrete cytokine SDF-1 which boots the proliferation of a number of cancer cell lines in culture including breast carcinoma cells. SDF-1 is capable of enhancing the survival of these cancer cells. In mice with genetic defects in macrophages, impaired metastases of breast-cancer cells to the lungs take place [62]. Stromal cells and their growth factors can provide selective prometastic advantage. For instance, TGF-β has the ability to induce the expression of various genes such as Angiopoetin-like (*ANGPTL4*) gene and enhance metastic activity to the lung in estrogen receptor negative ER^- breast cancer [63].

1.2.3 Stem Cells

Stem cells have the ability of self-renewal. Cell division in a stem cell leads to the production of at least one daughter cell that has the same characteristics of the parent. In general, stem cells are quiescent and may divide asymmetrically to generate another stem cell and one transit-amplifying cell. This provides continuation of the stem cell and the initial material for production

of differentiated cells. The maintenance of cell division and differentiation of stem cells possibly will lead to an understanding of the signaling pathways that are involved in cancer progression and then lead to yields new approaches for cancer treatment [64].

Cancer stem cells are capable of exponential proliferation, long-term proliferation, self-renewal, and multipotency. These result from the plasticity of stem cells that permits them to renewal several lineages of differentiated cells during homeostasis. Normal and cancer stem cells share the property of expression of the ATP-binding cassette (ABC)-G2 transporter ABCG2 [64] but ABCG2 does not express in the most non stem cells [65]. ABCG2 has the ability to pump out a variety of cells substrates such as cytotoxic drugs, using ATP energy. Cancer stem cells (CSCs) can be protected form cytotoxic drugs that are being used for cancer treatment by the high expression of ABCG2 transporters [64]. CSCs may able to contribute to therapeutic resistance [64]. The functional property of ABCG2 has been used to study mammary stem cells [64]. The identification of a population of cells known as the side population (*SP*) results from ABCG2 transporter activity and can be obtained using flow cytometric analysis [64]. Stem cells (SP cells) can be isolated without the need for cell surface marker analysis [65]. In breast, SP cells consist of 0.2-2% of epithelial tissue. When theses cells are injected into the cleared mammary fat pad, they generate both ductal and lobuloalveolar tissue [65].

1.2.3.1 Role Of Stem Cells In Tumorgenesis

The most important model of the several models that have been used to explain the origin and continued growth of tumors is the clonal evolution of tumors theory. This theory indicates that cancer generates from mutations that affect a few cells or a single cell and then leads to uncontrolled and unlimited proliferation of a population of cell [66]. The alteration of genes that result from the tumor progression leads to the activation of proto-oncogenes into oncogenes and inactivation of tumor-suppressor genes. Eventually, subtypes of cells in the tumor are capable of evading apoptosis; promoting self-sufficiency in growth signaling, tissue invasion and metastasis, and have limitless explicative potential [67]. The clonal evolution of tumors theory suggests also that mutations in various cells may lead to the selection of cells that have the ability to proliferate, seed new tumors and survive more than other cells. Furthermore, two other models the Stochastic and Hierarchical models, explain how tumors develop and progress

through unlimited cell division [64] and why not every cell in a tumor has the ability to regenerate the tumor [65]. The Stochastic Model predicts that every cell within the tumor is capable of being tumourigenic, while the hierarchical model suggests that only a limited number of cells have the tumourigenic capacity and the rest cannot generate the tumor on their own [64, 65]. The Hierarchical Model is in a concordance with the cancer stem cell hypothesis. This hypothesis proposes that cancer stem cells are the cells responsible for tumor self-renewal and not the differentiated cells that create the mass of the tumor [64]. The Hierarchical Model also predicts that it ought to be possible to separate tumor initiating cell (T-IC) which are capable of maintaining tumor growth from cells within the tumor, and form non T-IC. Purification of tumor cells into subfractions and a functional assay is required for resolution of the T-IC problem. This leads to the identification of cells that initiate tumor growth in vivo [65]. However, purification of solid tumor T-IC is not easy due to the lack of cell surface markers on this tumor. Cell surface markers make it possible for cell to be sorted [65].

1.2.3.2 Breast Cancer Stem Cells And Mammary Stem Cells

From epidemiology data on breast cancer incidence following radiation exposure, Little and Boice deduced that stem cells play a role in breast cancer [68]. Women who are exposed to radiation in late adolescence have a higher chance of getting breast cancer compared to women who are exposed to radiation at other ages. This indicates that adult mammary stem cells may accumulate genetic mutations that eventually lead to the development of solid tumors. It should be noted that stem cells are capable of resisting radiation as to they are slow cycling. Therefore, cells within tumors that are capable of surviving radiation may be breast cancer stem cells (BCSCs) that share the same property; that is the slow-cycling quality of stem cells [64]. Breast cancer initiating cells (BrCa-IC) or breast cancer stem cells (BCSCs) are slow-dividing and have a lowered capability to undertake apoptosis and a higher ability of DNA repair which lead these cells to be more resistant to traditional methods of cancer treatment such as chemotherapy and radiation [64, 65].

Some *In situ* observations have identified candidate stem cells that are estrogen receptor ER^+. fER^+ stem cells are important in adult mammary gland homeostasis [69]. However, ER^- stem cells have also been identified in mammary tissue and may represent the more primitive mammary stem cells. It has been observed that mammary reconstituting cells are ER^- and

(progesterone receptor) PgR^- [70]. Two thirds of breast cancer tumours are ER^+ and most of these tumours rely on oestrogen for growth. Therefore, breast cancer tumours that are ER^+ can be treated with hormonal therapy [71].

The identification of breast cancer initiate cells (BrCa-IC) or (BCSCs) that can be obtained using a reliable NOD/SCID xenoexplant assay. According to Al-Hajj et al, the injection of single suspensions of human breast cancer stem cell into the mammary fat pad of nonobese diabetic/severe combined immunodeficiency disease (NOD/SCID) mice, which are more immune-deficient than nude mice, causes these cells to grow tumors in NOD/SCID mice [64, 65]. Four cell surface markers including adhesion molecules CD44 and CD24 [65, 72], a breast/ovarian cancer specific marker B38.1 and epithelial specific antigen (ESA) with heterogeneous expression in breast cancer tissue can be used for the identification of BrCa-IC [65]. The results indicate that all mice injected with $CD44^+$, $B38.1^+$ or $CD24^{-/low}$ generates tumors while none of the $CD44^-$ and $B38.1^-$ injections can cause tumors.

In addition, lineage markers for all breast cancer tissue including hematopoietic, endothelial, mesothelial and fibroblast cells (Lin^+) are used to show that only breast cancer tissue (Lin^-) contains BrCa-IC. Lin^- $CD44^+$ $CD24^{-/low}$ are divided depend on the expression of epithelial surface antigen (ESA). It has been found that Lin^- ESA^+CD44^+ $CD24^{-/low}$(NOD/SCID) mice can cause tumors. However, ESA^- $CD44^+$ $CD24^{-/low}$ cells do not. The Lin^- ESA^+ $CD44^+$ $CD24^{-/low}$ cells represent a small subpopulation that account for 2% of unfractionated breast cancer cells and by using semiquantitative limiting dilution analysis proposed that the activity of BrCa-IC enriched by 50 fold in this fraction. The combination between cells sorting with functional assays demonstrates that breast cancer is heterogeneous and only BrCa-IC is capable of initiating human breast cancer. Moreover, BrCa-IC is capable of self-renewed and generating progeny that lose the ability to initiate tumor growth and require maturation markers. BrCa-IC share with normal stem cells the same properties which are self renewed and differentiated progeny [65]. Several markers have been used to identify BrCa-IC (Table 2) [64].

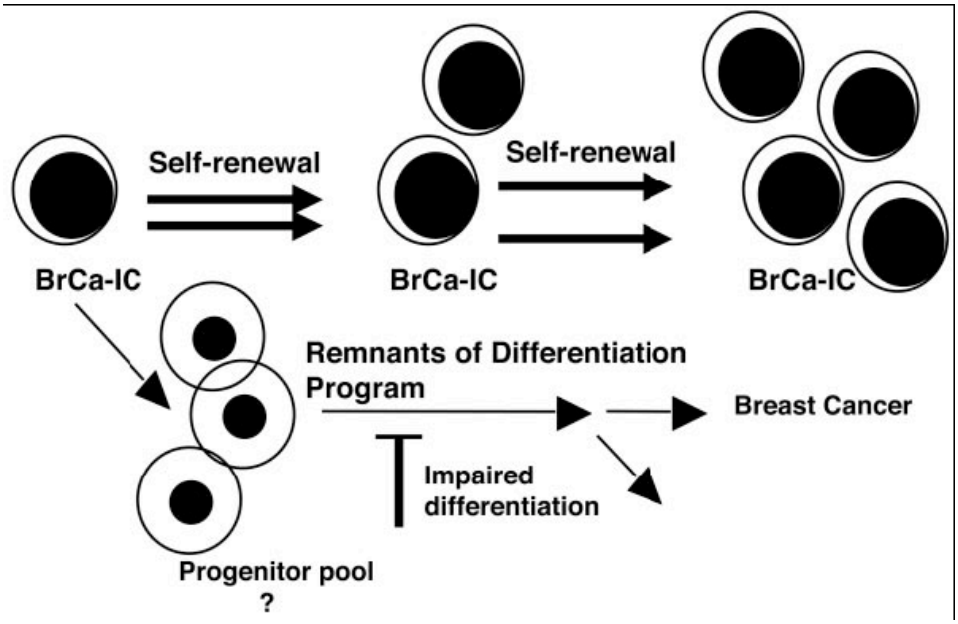

Figure 2: A model depicting the hierarchical organization of the breast cancer tumor. A rare and primitive breast cancer-initiating cell (BrCa-IC) maintains the breast cancer tumor [65].

Stem cells are responsible for the generation of the mouse's mammary gland. After implantation of a single cell (the mammary gland stem cell) (MGSC) into receiver mice, the entire mammary gland of the mouse can be regenerated. This type of cells has also the ability to generate ductal and lobuloalveolar lineages in the mouse (Figure 3) [65]. Breast SP cells have a high level of expression of the hematopoietic stem cells (HSC) marker, Sac-1. After injection, cells that have Sac-1^{+} marker are capable of initiating mammary gland regeneration, while Sac-1^{-} do not contain any MGSC. Flow cytometry has been used for phenotype and isolating putative mammary stem cells and BCSCs cell populations [65].

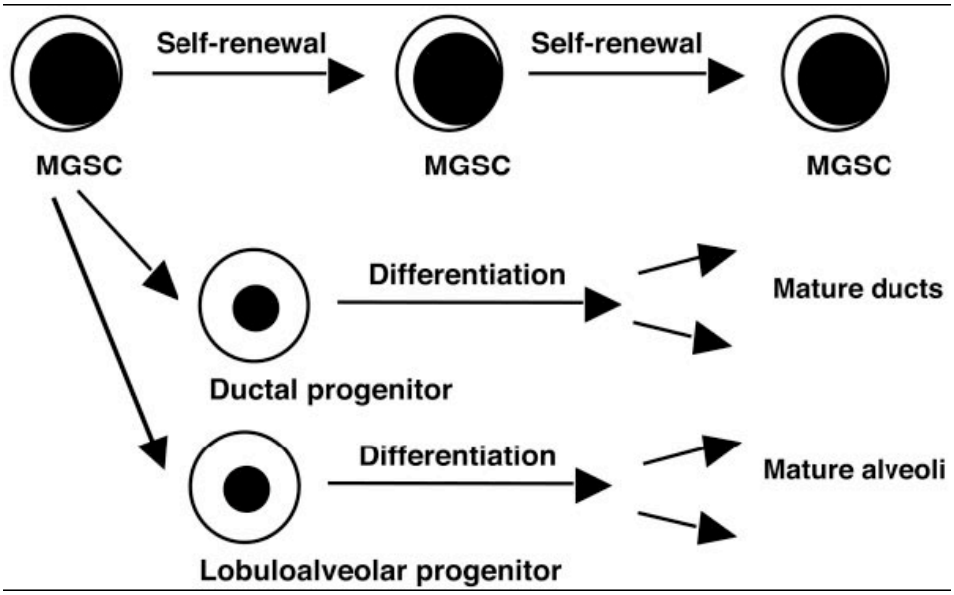

Figure 3: Schematic representation of the organization of the mammary gland. This model proposes that only mammary gland stem cells possess a high self-renewal capacity and the developmental capacity to produce lineage-committed progenitors [65].

Table 2: It shows cells surface markers that expressed by breast cancer stem cells [64].

Factor	Characteristics	Reference(s)
Cell surface markers that are expressed by putative breast cancer stem cells		
ABCG2	ABCG2 (ATP-binding cassette G2) is a class of drug transporters capable of pumping cytotoxic drugs out of the cell.	[6]
CD44	CD44 is involved in cellular adhesion, motility, and metastases.	[34,35]
CD10	CD10 is a common acute lymphoblastic leukaemia antigen that is overexpressed on many tumours.	[39]
EpCAM/ESA	Epithelial cell adhesion molecule/Epithelial surface antigen is expressed on mammary tissue and tumours.	[39]
CD29 (β1-integrin)	CD29 is a membrane receptor involved in cell adhesion and metastatic diffusion of tumour cells.	[9]
CD49f (α6- integrin)	CD49f is involved in basal and endothelial cell distribution and is a candidate stem cell marker.	[39]
CD133 (prominin-1)	CD133 is a cell surface glyoprotein with an unknown function in cancer stem cells and its expression is documented for various types of cancer.	[116,117]
ALDH1	Aldehyde dehydrogenase-1 plays a role in the differentiation of stem cells and its activity predicts poorer clinical outcomes.	[112]

CXCR4	CXCR4 is a chemokine receptor involved in metastasis and its expression is increased in mammospheres.	[44,45]
ER	Oestrogen receptor is expressed on breast cancer cells, mammary progenitors, and breast cancer stem cells.	[54,59]
Signalling pathways that play a role in cancer stem cells		
Delta/Notch pathway	This pathway is involved in cell fate development and is expressed in stem cells and early progenitor cells.	[60]
Notch-4	Notch-4 plays a role in mammary development and its overexpression has been shown to promote mammary tumours.	[45,60,61]
Wnt signalling pathway	This pathway is involved in stem cell self-renewal and its overexpression can lead to epithelial and mammary tumours.	[27,63]
β-catenin	β-catenin is a downstream target of the Wnt pathway. A pro-oncogenic role has been described.	[63]
Hedgehog/Patched pathway	This pathway is involved in embryonic growth and cell fate determination.	[64]
PITCH	A receptor for the Hedgehog signalling family, PITCH has been connected to early embryonic tumourigenesis.	[64]
EGFR	Epidermal growth factor receptor signalling has been found to be upregulated in breast cancer stem cells and may be required for mammosphere formation.	[77,111]

1.2.3.3 Targeting Stem Cells For Elimination

Cancer therapy focus on targeting specific markers on tumor cells in order to stop the development of the tumor. These markers are over expressed or mutated and often stand for genes, proteins or pathways considered to be crucial for the development of the tumor. For instance, Herceptin (called traztuzamab) targets the HER2 oncogene. A number of studies show that *HER2* gene over expression accounts for around 20- 30% [6, 7, 64, 73] of human breast cancers and is associated with aggressive metastatic disease. HER2 has the ability to regulate several signaling pathways such as PI3-K/Akt. The contribution of this signaling in causing aggressive tumors is still unknown [73].

The stem/progenitor cell population can be identified using Aldefluor assay or immunohistochemistry that are capable of determining the quality of increased expression of the enzyme aldehyde dehydrogenase (ALDH1) in both of the normal mammary gland and mammary carcinomas. Ginestier et al observed that there is a significant correlation between over expression of HER2 and expression of ALDH1 that is a stem cells marker in a large number of

breast cancer patients [74]. The regulation of mammary stem cells population may be affected by the over expression of HER2. This over expression leads to a rise in the population of stem/progenitor cells of both normal and malignant mammary cells. CSCs or tumor stem cells are able to mediate tumorgenesis, invasive and metastasis as an effect of HER2 over expression.

There is a correlation between the overexpression of HER2 and Akt phosphorylation. HER2 over expression leads to the enhancing of the phosphorylation of Akt in Aldefluor-positive HER2 stem cells. Trastuzumab has the ability to inhibit the overexpression of HER2 and AKT phosphorylation in sensitive but not in resistant cells. Hasan et al noted that there is no measurable effect on phospho-Akt and on phospho-HER2 in trastuzumab resistant cells. They propose that the PI3-K/Akt pathway plays a crucial role in mediating the effects of HER2 signaling in breast cancer. PI3-K pathway has been identified as the main determinant of trastuzumab resistant in breast cancer. Akt signaling also has a role in stem cell self-renewal. New targets for cancer prevention and therapy can be obtained if the components of these pathways are identified [73].

1.2.4 Breast Cancer Implications

Several treatments have been used to treat breast cancer including surgery, radiation and systemic therapy, which include hormonal therapy, chemotherapy and biological therapy. However, chemotherapy leads to side effects and the appearance of drug resistance. An understanding of the cellular signaling pathway of breast cancer might lead to the development of rational a therapeutic treatment. As mentioned, Trastuzumab is a humanized monoclonal antibody against HER2. It was the first HER2-targeted biologic agent permitted for the treatment of breast cancer (Figure 4) [6]. Treatment of breast cancer is based on the inhibition of tyrosine kinase HER2-receptor [75]. Trastuzumab, which works as a blocking antibody, is approved for the adjuvant treatment of HER2-overexpressing [76]. The survival of patients with HER2-positive metastatic breast cancers has been enhanced when the combination of trastuzumab and chemotherapy have been used. However, the development of resistance to trastuzumab and its high cost restrict its therapeutic use. Several small molecules have been developed such as erlotinib, gefitinib, lapatinib and canertinib, target HER1 tyrosine kinase activity. Furthermore, farnesyl transferase inhibitors target Ras, and rapamycin and its analogues inhibit mammalian

target of rapamycin (mTOR). These molecules show limited success when they are used alone; but they become more efficient when they are combined with trastuzumab, antiestrogens or aromatase inhibitors [6].

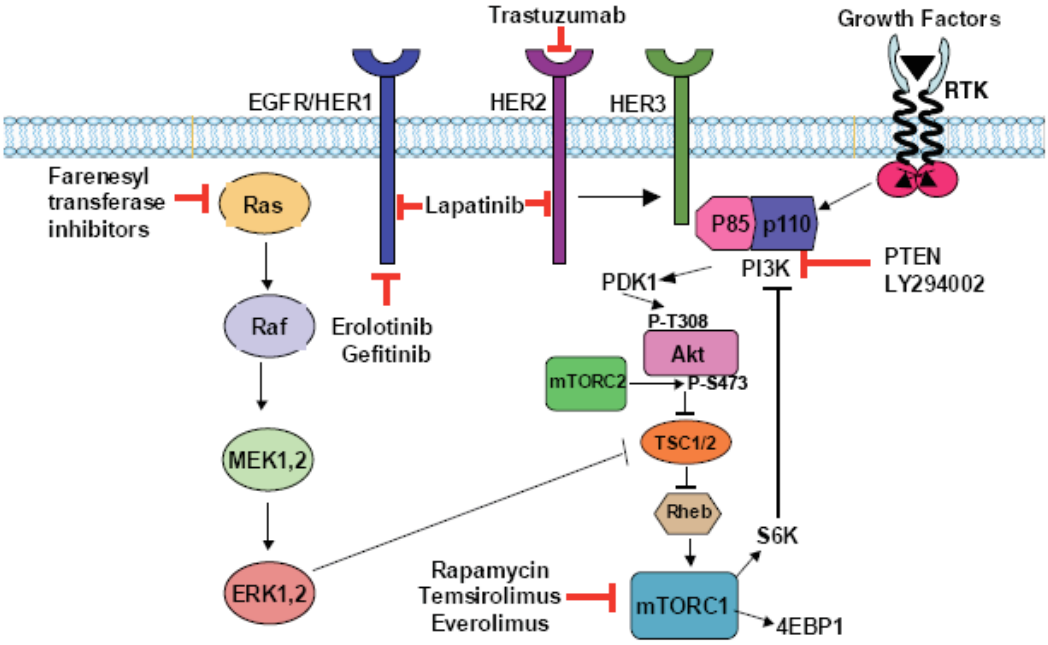

Figure 4: Akt signaling and targets for breast cancer therapy [6].

The key mediator of cell signaling during development is Type I Receptor Tyrosine kinases (RTK-I). This is associated with cancer mestastasis [77, 78]. The over expression of HER1 and HER2 genes, which are members of RTK-I implicate in causing breast cancer [2, 3]. Over expression of HER1 and HER2, which are preferred interacting partners for dimerization in breast cancer (Figure 5), is associated with a poorer clinical prognosis and predicts a bad response to endocrine therapy [8].

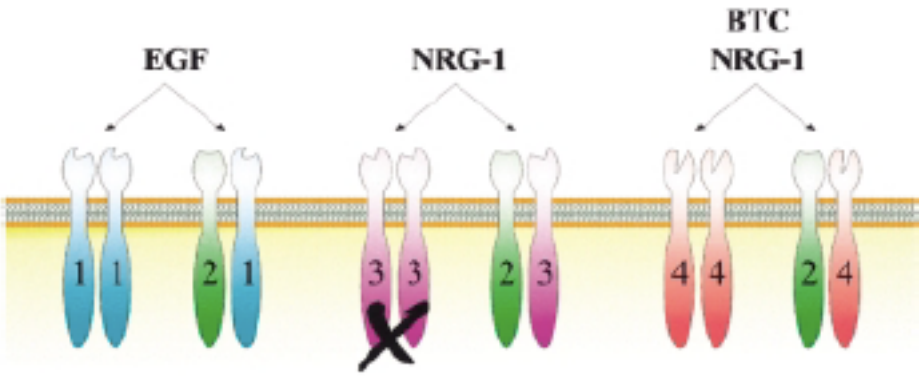

Figure 5: HER2 is the preferred dimerization partner for the other HERs. Ligand binding to HER1 (EGF), HER3 (Neuregulin-1 (NRG-1)) or HER4 (NRG-1, Betacellulin (BTC)) induces the formation of receptor homodimers and HER2-containing heterodimers. HER3 homodimers do not signal (indicated by the X), since the receptor has impaired kinase activity [79].

Cohen isolated EGF from the mouse sub-maxillary gland. It is an effective mitogen for a number of cell types in vivo or culture [13, 80]. EGF consists of a 53 amino acid polypeptide [81]. EGF binds to specific high affinity cell surface receptors and stimulates replication of a number of cells. It stimulates a membrane associated with protein kinase that leads to the phosphorylation of tyrosine residues at on the cytoplasmic tails of the receptor pair and subsequent stimulation of downstream signaling pathways such as PI3K/Akt, Ras/ Raf/Mek/Erk, PLCγ 1/PKC, and STATs pathways (Figure 6) [82].

The high expression of *HERs* in breast cancer cells contributes to aggressive and chemotherapy resistant tumours. Therefore, recent therapies aimed at overcoming this resistance have focused on suppressing HER signaling. This suppression will eliminate cell proliferation or survival signals and trigger tumour regression [83]. As mentioned, Trastuzumab has been used in treating HER2-positive breast cancers. However, Trastuzumab only provides an overall response rate of 15% in advanced metastatic breast cancer [7]. A number of studies have confirmed that the addition of Trastuzumab to standard chemotherapy may enhance the survival rate from 20.3 to 25.4 months [84]. Therefore, the invention of new and novel treatments for cancers that over-express HERs is required urgently. In our experimental approach, A431 cells, derived from epidermoid carcinoma, are used as a model to study breast cancer. This cell line over expresses *HER1* and when treated with a high dose of EGF will undergo apoptosis via the activation of HER1 signaling [11-13].

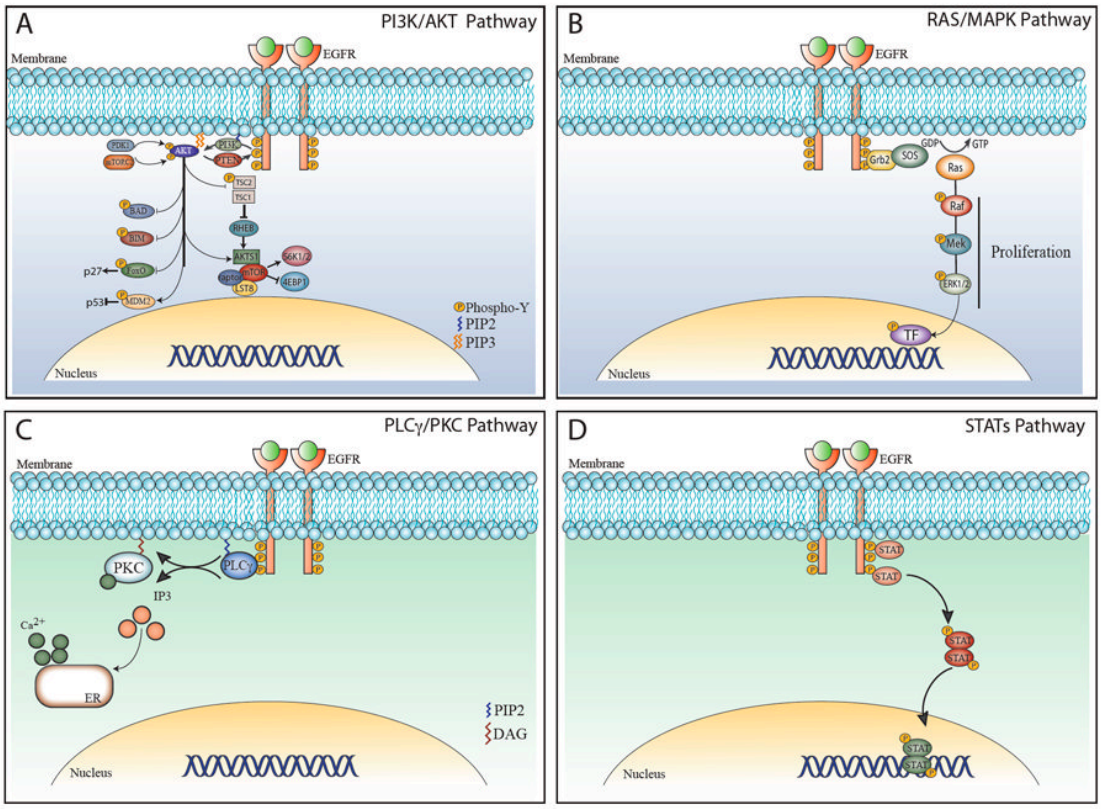

Figure 6: Classical HER1 Signaling [82].

1.2.5 Experimental Approach

A431 cells are called epidirmoid carcinoma cells. They express an unusually high density of EGF receptors. Treating A431 cells with a high dose of EGF leads to the inhibition of A431 cells growth and replication [11, 13, 85], despite EGF having the ability to stimulate replication of many deferent cell types [13]. Inhibition of A431 growth by EGF may be because the huge energy expenditure necessary for receptor phosphorylation, internalization, degradation and new receptor synthesis which must occur on a large scale in comparison to other cell lines with fewer receptors [86]. Moreover, excessive ligand binding would increase tyrosine kinase activity. This results in deregulated growth signaling, leading to programmed cell death [87]. In addition, the second chapter (First paper) suggests that EGF induced apoptosis in A431 cells happens because of multiple pathways [88]. At low EGF concentrations, A431 cells perhaps do not undergo apoptosis because there is little energy expenditure necessary for receptor phosphorylation, internalization, degradation and new receptor synthesis [86] and the reduction in EGF-stimulated tyrosine kinase activity seems to be essential for the escape from growth inhibition and

consequent apoptosis [87].

A431 cells respond to EGF by rapid change in cell morphology with the formation of ruffles, filopodia and inducing cell rounding. EGF also has the capability to induce clustering of EGF receptors and has a rapid effect on pinocytotic activity [11, 13]. The results suggest that EGF is responsible for stimulation protein kinase activity in vitro. This is a crucial component of the mechanism of EGF action [13, 85]. In protein tyrosine kinase (PTK) pathways, the binding of EGF factor to HER1 in cell surface contributes to activation of the receptor tyrosine kinase or is known as receptor associated tyrosine kinase. This activation leads to an activation of the STAT (signal transducer and activator of transcription) signaling pathway which can plays a crucial role in cell differentiation, cell cycle control and development [14], but can also induce apoptosis in A431cells [12, 14].

Apoptosis (programmed cell death) is a mode of cell death that can be recognized by cellular and biochemical hallmarks such as activation of caspases, chromatin condensation, release of apoptogenic factors, and membrane blebbing [89]. Apoptosis is used by multicellular organisms to eliminate unwanted cells and maintain homeostasis during development [90]. Deregulation of apoptosis causes many diseases including cancer [91]. Various strategies are used in clinical oncology including chemotherapy, gamma irradiation, suicide gene therapy or immunotherapy for killing cancer cells. These strategies have been associated with activation of apoptosis signal transduction pathways in cancer cells [92]. In many cases, these strategies are capable of activating caspases, a cysteine protease family. Caspases act as common death effector molecules in different forms of cell death, are synthesized as inactive forms and upon activation, cleave proteins at aspartate residues [93]. They are capable of cleaving a large number of substrates in the cytoplasm and nucleus [94]. Caspases are also able to activate each other, which causes amplification of caspase activity through a protease cascade. A number of studies have discussed two possible intrinsic and extrinsic pathways in apoptosis that have the ability to initiate the activation of caspases at different entry points [92, 95]. The extrinsic pathway is identified as a death receptor pathway, while the intrinsic pathway is a mitochondrial pathway [95, 96]. There are more than 20 death receptors of the tumor necrosis factor (TNF) receptor superfamily, such as CD95 (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL)

receptors [97]. Stimulation of cell surface death receptors such as Fas leads to activation of caspase-8 which in turn cleaves downstream effector caspases such as caspase-3 (Figure 7) [92]. A mitochondrial pathway is initiated by the release of pro-apoptotic proteins such as cytochrome *c*, Smac (second mitochondriaderived activator of caspase) /DIABLO (direct inhibitor of apoptosis protein (IAP)-binding protein with low PI), and the serine protease HtrA2/Omi from the inter-membrane space into the cytosol. These proteins activate the caspase dependent mitochondrial pathway. Cytochrome c binds and activates Apaf-1 as well as procaspase-9, forming an "apoptosome" which in tune activates caspase-3. Whereas HtrA2/Omi and Sam/DIABLO play a role in caspase activation in a caspase-dependent way by inhibiting IAP (Inhibitors of apoptosis proteins) activity while HtrA2/Omi also plays a role in a caspase-independent way as a protease [98]. Furthermore, the cleaved pro-apoptotic form of Serpinb1 (L-DnaseII) is capable of inducing apoptosis in a caspase-independent fashion in response to metabolic stress [99, 100]. L-DNaseII is translocated to the nucleus where it degrades DNA [101]. Cytoskeleton reorganization may lead the induction of cell death in caspase independent fashion [102].

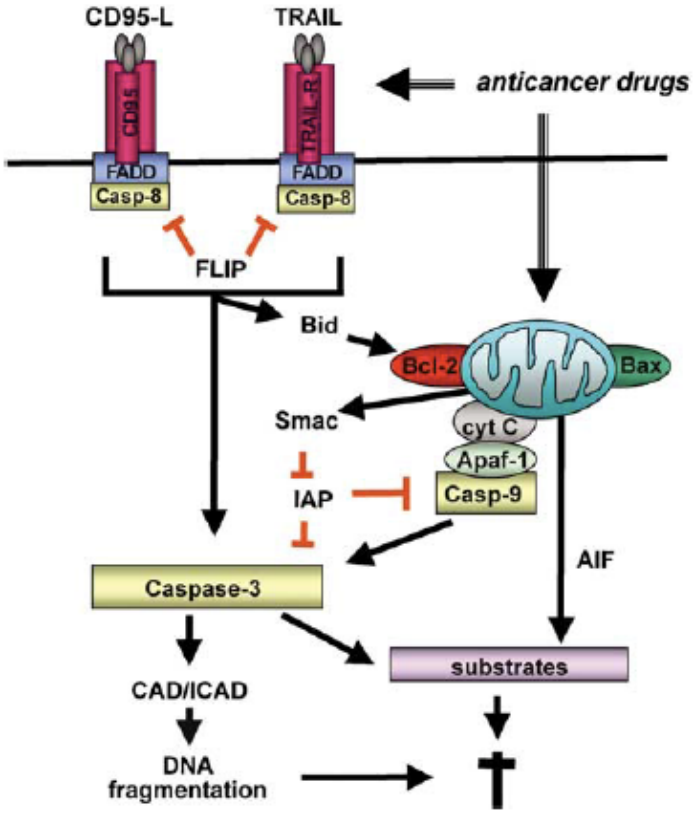

Figure 7: Apoptosis signaling pathways. Apoptosis pathway can be initiated through extrinsic (receptor) and intrinsic (mitochondrial) pathways [92]. CD95 (APO-1/Fas), TNF-related apoptosis-inducing ligand (TRAIL) receptors, FADD (Fas (TNFRSF6)-associated via death domain), FLIP (CASP8 And FADD-Like Apoptosis Regulator), BID (BH3 interacting domain death agonist), BCL-2 (BCL2-Associated Agonist Of Cell Death), BAX (BCL2-associated X protein), cyt C (Cytochrome c), APAF1 (apoptotic peptidase activating factor **1**), CASP9 (caspase 9, apoptosis-related cysteine peptidase), Smac (second mitochondriaderived activator of caspase) /DIABLO (direct inhibitor of apoptosis protein (IAP)-binding protein with low PI), IAP (Inhibitors of apoptosis proteins), AIF (Apoptosis-Inducing Factor) and CAD/ICAD (Caspase-Activated DNase/ Inhibitor of Caspase Activated DNase).

In our model A431 cells, EGF activates STAT1 and STAT3 proteins. Activated STAT proteins translocates from the cytoplasm to the nucleus in order to regulate responsive genes such as cyclin-dependent kinase (CDK) inhibitor p21 $^{WAF1/CIP1}$. P21 $^{WAF1/CIP1}$ is on of the family of CDKs members that control the cell cycle and is responsible for cells growth arrest [12, 14]. Also, activation of the STAT signaling pathway can induce apoptosis via the induction of ICE caspase 1 (interleukin-1βconverting enzyme [ICE]) gene expression (Figure 8) [14]. Because the genetic network through which HER1 can induce apoptosis is not known at the present time, this work in this thesis was carried out to identify the genetic network and downstream components though which HER1 induces apoptosis. These downstream components are likely to be useful targets for chemotherapy.

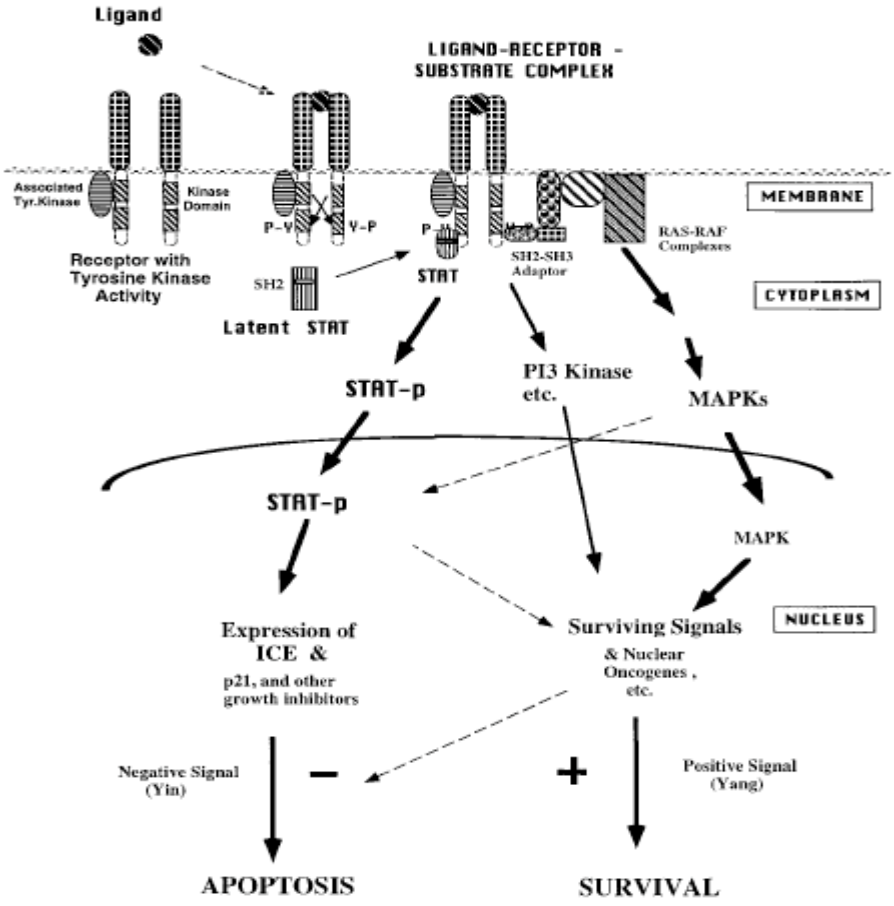

Figure 8: yin (negative) and yang (positive) signaling model of PTK-STAT induced apoptosis. Simultaneously, a mitogenic pathway, such as the Ras-MAP kinase pathway, which regulates the expression of genes, such as oncogenes, that positively control cell growth and survival is also activated [14].

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CHAPTER TWO

Combined Gene Expression and Proteomic Analysis of EGF Induced Apoptosis in A431 Cells Suggests Multiple Pathways Trigger Apoptosis.

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CHAPTER THREE

MicroRNAs are part of the regulatory network that controls EGF induced apoptosis, including elements of the JAK/STAT pathway, in A431 cells.

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MicroRNAs are part of the regulatory network that controls EGF induced apoptosis, including elements of the JAK/STAT pathway, in A431 cells.

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Abstract

miRNAs are known to regulate gene expression and in the context of cancer have been shown to regulate metastasis, cell proliferation and cell death. In this report we describe potential miRNA regulatory roles with respect to induction of cell death by pharmacologic dose of Epidermal Growth Factor (EGF). Our previous work suggested that multiple pathways are involved in the induction of apoptosis, including interferon induced genes, cytokines, cytoskeleton and cell adhesion and TP53 regulated genes. Using miRNA time course expression profiling of EGF treated A431 cells and coupling this to our previous gene expression and proteomic data, we have been able to implicate a number of additional miRNAs in the regulation of apoptosis. Specifically we have linked miR-134, miR-145, miR-146b-5p, miR-432 and miR-494 to the regulation of both apoptotic and anti-apoptotic genes expressed as a function of EGF treatment. Whilst additional miRNAs were differentially expressed, these had the largest number of apoptotic and anti-apoptotic targets. We found 5 miRNAs previously implicated in the regulation of apoptosis and our results indicate that an additional 20 miRNAs are likely to be involved based on their correlated expression with targets. Certain targets were linked to multiple miRNAs, including PEG10, BTG1, ID1, IL32 and NCF2. Some miRNAs that target the interferon pathway were found to be down regulated, consistent with a novel layer of regulation of interferon pathway components downstream of JAK/STAT. We have significantly expanded the repertoire of miRNAs that may regulate apoptosis in cancer cells as a result of this work.

Introduction

The epidermal growth factor receptor (HER1) and HER2 are members of the receptor tyrosine kinase type one (RTK-I) family. Activation of these receptors plays crucial roles in survival, migration [1], development, proliferation and differentiation [2]. Epidermal growth factor (EGF) and other ligands binds to HER1 and forms homodimers or hetrodimers with HER2 [2]. Activation of HER 1 signalling usually induces either proliferation or cell survival [3], a fact that has resulted in a strategy that targets RTK-I mediated survival signals in cancer [4-10]. However, there are significant and reproducible reports of EGF inducing apoptosis in cell lines such as A431 that over-express RTK-I [11-17]. The gene expression and protein profiles of these cells were characterised after EGF treatment in order to globally survey the induction of apoptosis[18]. As a result of this study, multiple novel regulatory sub-networks /pathways mediating EGF induced apoptosis were observed. Because MicroRNAs (miRNAs) are known to regulate pathways governing cancer cell survival, differentiation and metastasis we expected that they might be important regulators of the above gene expression/protein sub-networks.

MiRNAs are a major class of gene regulators that participate in large scale modulation of gene expression [19, 20]. MiRNAs are noncoding RNA molecules transcribed as nascent primary miRNA transcripts (Pri-mRNA) by RNA polymerase II. After Pri-mRNA transcripts are processed into miRNA precursors (pre-miRNA) by Drosha and Pasha, pre-mRNAs are exported from the nucleus to the cytoplasm by exportin 5 and subsequently processed by Dicer into mature miRNAs. These miRNAs can incorporate into the RNA-induced silencing complex (RISC) to target protein-

coding messenger RNA (mRNA) and inactivate gene expression by either mRNA degradation or inhibition of translation [21-24]. MicroRNAs have recently been described as novel tools for cancer diagnosis and may help identify and validate cancer targets [22-24]. A recent report shows that some miRNAs such as miR-17-5p/miR-20a can suppress breast cancer cell proliferation via a conserved 3'-UTR miRNA-binding site [21]. In addition, miRNAs regulate the expression of target genes that can act as a controllers of growth, development, differentiation, cancer development and progression [25-27]. Furthermore, miRNAs are key regulators of apoptosis in cancer because they can act as positive regulators of apoptosis by acting as negative regulators of anti-apoptotic mRNAs and as negative regulators of apoptosis by acting as negative regulators of pro-apoptotic mRNAs [28].

Recently, we found that when a high dose of EGF is used to induce apoptosis in A431 cells that multiple gene expression and protein expression sub-networks are stimulated, including interferon response, cytokine signalling, cytoskeleton and cell adhesion pathways. Specifically we found that between 3h and 12h post-EGF treatment significant changes in gene expression occurred in the lead up to caspase induced apoptosis. In order to provide a deeper understanding of these regulatory mechanisms, it is essential to build an integrated genetic regulatory network that includes post-transcriptional (miRNA) regulatory interactions.

In this study, we have identified novel interaction regulatory networks based on the crosstalk between miRNAs and mRNA/protein that resulted in the induction of apoptosis in A431 cells. We have also identified a number of miRNAs that may play an important role in the regulation of apoptosis in A431 cells after EGF treatment.

Materials and Methods

Cell Culture and Treatment

A431 (The epidermoid carcinoma cell line) cell line was purchased from the ATTC (Manassas,VA). Cells were cultured at 37°C in a humidified 5% CO2 atmosphere. Dulbecco's modified Eagle's medium, 10% fetal calf serum, and 4 mM L-glutamine (Cambrex Bio Science). After 24h serum-free, in addition to control (no EGF treatment), cells were treated with 100 ng EGF concentrations and sampled at 3h and 12h after treatment [18].

Microarray Analysis for miRNA Expression in A431 cell line after EGF treatment.

Total RNA were extracted from A431 cells using the mirVana miRNA isolation kit (Ambion, Inc., Austin, TX, USA), according to the manufacturer's instruction. RNA's concentration was determined by Nanodrop (ND1000) spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and its purity was determined by bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). We performed comprehensive expression profiling of mature miRNA using the Affymetrix GeneChip 2.0 miRNA Array (Affymetrix, Santa Clara, CA). Labeling of 1μg of total RNA samples was performed using the FlashTag Biotin RNA labeling kit (Genisphere Inc., Hatfield, PA) according to manufacturer's instruction. Using an Affymetrix GeneChip Fluidics Station 450, the arrays were washed and stained and then scanned with an Affymetrix GeneChip scanner (3000 7G). The CEL files containing the raw Affymetrix 2.0 microRNA array intensity data were processed using the Bioconductor tools that were used for image analysis, data import, background adjustment, normalization (based on

RMA algorithm), summarization, and quality assessment [29, 30]. Cancer cell line (no EGF application) and two time points after EGF application (3h and 12 h) were assumed as treatments. For each time points, RNAs were extracted from 3 samples as replications. Differential gene expression analyses were performed using linear regression models in the limma package [31]. For transcript analysis (microarray data), Log2 ratio values of each time point verses control (cancer cell line) were assumed as fold change. Those having a Bayesian t-test with adjusted p-value with false discovery rate <0.1 and fold change ≥ +1.5 or fold change ≤ -1.5 were accepted as significant differentially expressed genes.

Microarray Analysis for mRNA Expression in A431 cells after EGF treatment.

Cell culture, mRNA isolation and microarray analysis described in [18].

Proteomic Analysis of A431 cells after EGF treatment.

Cell culture, protein isolation and proteomic profiling described in [18].

Target prediction of significantly expressed microRNA

Several methods were used to predict targets of significantly expressed microRNAs including miRBase (http://www.mirbase.org/), and TargetScan (www.targetscan.org/). Targets of significantly expressed microRNAs were then matched with significantly expressed genes (using our microarray data) and significantly expressed proteins (using our proteomics data) in order to obtained only genes and proteins in our data potentially regulated by microRNA(s).

Reactome functional interactions (FI) Cytoscape

Correlations between genes/proteins involved in the same functional interactions (FIs) were carried out using The Reactome FI Cytoscape plugin (Pearson correlation). The correlations were then used as input for the Reactome FI Cytoscape plugin (Markov cluster algorithm) to generate a sub-network for a list of selected network modules based on module size and average correlation [32].

Gene and protein Classification According to Gene Ontology

PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification software was used to assign protein classes to identified genes and proteins [33].

Results

MiRNA expression.

Our analysis of the miRNA time course expression values, based on a False Discovery Rate of 10% yielded 37 miRNAs that were differentially expressed between the time of EGF treatment and either 3 hours or 12 hours post-EGF treatment (Table 1). Of the 37 differentially expressed miRNAs about two thirds decreased at 3 or 12 hours post EGF treatment, and the remaining third increased in expression. The changes between 0h and 3h and 0h and 12h were very consistent with only two miRNAs flipping from either increased expression at 3h to decreased expression at 12h or vice versa (Figure 1A). The two miRNAs that flipped expression pattern with respect to untreated cells were miR-432 (-2.04 fold change at 3h, 2.03 fold change at 12h) and miR-499-5p (2.26 at 3h, -1.22 at 12h).

MiRNA targets.

Because miRNAs can target multiple mRNAs we expected the total number of target genes regulated by these miRNAs to be greater in number. In order to identify likely target transcripts we identified all possible target genes using both TargetScan and miRBase and then determined which targets were differentially expressed within our gene-expression and proteomic data. As a result of this analysis we identified 165 unique differentially expressed genes at both 3 and 12h post EGF treatment in both our gene-expression data and proteomic data. In order to identify which targets might regulate apoptosis, we carried out an exhaustive manual literature search of all differentially expressed targets in order to annotate them with respect to possible pro or anti-apoptotic functions. Within the gene expression data set, 77 targets were differentially expressed at 3h post EGF treatment, with 17 of these annotated as having anti-apoptotic function, 20 annotated as pro-apoptotic and 2 annotated as both pro and anti-apoptotic. At 12h post EGF treatment there were 99 differentially expressed targets (some of which were shared with 3h post EGF) and 17 of these were anti-apoptotic, 19 pro-apoptotic and 1 both anti and pro-apoptotic (Supplementary Table 1). Within the proteomic data set we identified the same 12 targets at both 3h and 12 post EGF and of these 1 was anti-apoptotic and 6 were pro-apoptotic (Supplementary Table 2).

Because most of the differentially expressed targets had no previous link to apoptosis we used PANTHER [33] to classify our targets from both gene-expression and proteomic data with respect to function (Table 2). Our gene-expression targets fell into 25 classes including signalling molecules, receptors, transmembrane receptors,

transcription factors, cytoskeleton, cell-adhesion and extra cellular matrix. Our protein targets fell into 12 classes, including cytoskeleton and chaperone, a class not found in the gene-expression data. In order to visualise which targets might be acting together to regulate apoptosis we constructed correlation networks for gene-expression and proteomic data [18] and then added miRNAs based on their known targets in the networks.

MiRNAs and network regulation.

We used the Reactome Functional Interaction plug-in from Cytoscape [32] to construct correlation networks based on gene-expression or proteomic data. The correlation networks for gene expression differed significantly in complexity between 3h and 12h post EGF treatment, with a reduction in network complexity as judged by fewer nodes. As reported previously [18], at 3h (Figure 2A) we observed correlated changes in gene expression for a variety of mRNAs, including immediate early response genes, cytokines, cytokine signalling suppressors, interferon response genes and cell adhesion genes. When we added our miRNAs to the network based on the presence of known targets, we found 15 miRNA targets regulated by 16 miRNAs, 6 of which regulated 2 or 3 targets for a total of 24 edges connecting miRNAs to targets. In addition, 6 targets were regulated by more than one miRNA and miR-145 had 4 targets. Furthermore, we found that a greater proportion of anti-apoptotic genes were targets for miRNAs. Specifically, 9 of 21 anti-apoptotic genes were miRNA targets compared to 2 of 13 pro-apoptotic genes. Of the 6 targets regulated by more than one miRNA, 5 were anti-apoptotic (SOCS3 (suppressor of cytokine signaling 3), PM1(transmembrane protein 11), JUNB (jun B proto-oncogene), ITGA5 (integrin, alpha 5) and GRB7 (growth factor receptor-bound protein 7) with JunB an anti-

apoptotic target for 4 miRNAs. CBLB (Cbl proto-oncogene, E3 ubiquitin protein ligase B)was the only pro-apoptotic gene linked to 2 miRNAs.

Because negatively correlated miRNA/target pairs provide more compelling evidence of potential regulation we looked for miRNA/mRNA pairs that had negatively correlated fold changes. We found 14 miRNA/target edges that were consistent with 14 negatively correlated miRNAs and 8 mRNAs, of which 2 were pro-apoptotic and 6 were anti-apoptotic. However, at 12 h post-EGF treatment (Figure 2B) the network was much simpler with only 8 miRNAs connected by 8 edges to 5 targets (of 11 genes). Some of the edges were more consistent with a regulatory function, and 3 described miRNA/mRNA relationships that were negatively correlated. Two of these 3 genes were pro-apoptotic targets, SORT1 (sortilin 1) and TP53 (tumor protein p53), with SORT1 linked to 3 miRNAs. In fact, SORT1 mRNA actually increased from 3hrs in spite of being targeted by 3 miRNAs, 2 of which increased and one decreased from 3 hrs. The remaining mRNA was an anti-apoptotic target, MYB (v-*myb* myeloblastosis viral oncogene homolog (avian), targeted by a single miRNA (miR-134). It is worth pointing out that miR-663 targeted MCM5 (minichromosome maintenance complex component 5), which encodes a chromatin binding protein that may regulate the cell cycle [34]. MCM5 is part of a smaller, highly connected cluster that includes POLA1 (polymerase (DNA directed), alpha 1, catalytic subunit), MCM3 (minichromosome maintenance complex component 3) and MCM6 (minichromosome maintenance complex component 6) all involved in DNA replication.

We also found miRNAs targets in our protein expression correlation network (Figure 3). Unlike the gene expression sub-networks, at 3h (Figure 3A) and 12h (Figure 3B) post EGF treatment the same sub-networks were found for protein expression.

Overall we observed 7 miRNAs linked by 7 edges to 5 targets. At 3 h post-EGF all targets decreased in abundance, while at 12 h, 2 targets had increased in abundance. One sub-network, which included MIR-134 as a regulator of BANF1 (Barrier to autointegration factor 1), thought to function as a regulator of nuclear assembly, was correlated with the expression of PPIA (peptidylprolyl isomerase A (cyclophilin A)) an enzyme known to catalyse protein folding and known to participate in the induction of apoptosis [35], and XRCC6 (x-ray repair cross complementing protein 6) a DNA binding helicase capable of interacting with transcription factors but also known to protect cells against oxidative stress and apoptosis [36]. The other sub-network included primarily cytoskeletal proteins, with 4 targets, three of which, CAP1 (CAP, adenylate cyclase-associated protein 1), CFL1 (Cofilin 1) and ACTB (Actin, beta) are cytoskeletal components, and the remaining target TCP1 is known to regulate the folding of ACTB and TUBA1B (Tubulin, alpha 1b). Both ACTB and CFL1 were targets for two miRNAs, and CAP1, CFL1 and ACTB are known to be part of an interaction network regulating cytoskeletal polymerisation. Five of the 8 proteins in this sub-network are known to be pro-apoptotic and of these 8, both CAP1 and TCP1 increased in abundance from 3h to 12 h post EGF.

Overlap of gene-expression and proteome miRNA targets.

The overlap between the gene-expression data and the proteome data is small, with only 4 loci common to both sets, accounting for about 8% of the proteins and about 1% of the mRNAs [18]. However, the proportion of miRNAs that had targets in both the gene-expression data and the proteome data was higher. Of the 21 miRNAs (Table 3) with targets in either the gene-expression or proteome sub-networks, 14 had targets found only in gene-expression sub-networks, 1 had a target only found in

protein sub-networks and 6 had targets that were found in both gene-expression and protein sub-networks. However, no miRNA that had targets in both sets shared common targets across the two sets.

Negatively correlated miRNA/mRNA pairs.

If miRNAs were regulating either mRNA or protein expression in our experiment, we might expect that miRNAs that increased in expression would be associated with targets that decreased in expression and *vice versa*, leading to negatively correlated miRNA/target pairs. In our data a number of miRNAs showed negatively correlated changes in expression with targets known to regulate apoptosis (Tables 4 and 5), so these miRNAs and targets appear to be better candidates for further investigation. Within this negatively correlated miRNA/target set BTG1 (B-Cell Translocation Gene 1, Anti-Proliferative), which has anti-proliferative function and has been shown to induce apoptosis [37], increased in expression while the two potentially regulatory miRNAs (miR-596 and miR-1231) decreased in expression. ID1 (inhibitor of DNA binding 1, dominant negative helix-loop-helix protein) has been reported to induce apoptosis via P38 MAP Kinase [38] and increased in expression while the three miRNAs that target it (miR-494, miR-602 and miR-663) decreased in expression. IL32 (interleukin 32) also increased in expression and is known to induce apoptosis and was potentially regulated by miR-663 and miR-675, which decreased in expression. Finally, NCF2 (neutrophil cytosolic factor 2) also increased in expression and is known to induce apoptosis and was potentially regulated by miR-494 and miR-2861. Interestingly, IRF1 (interferon regulatory factor 1) a transcription factor of the interferon induced pathway associated with apoptosis also increased in expression and was targeted by Mir-2861 which decreased in expression. One particular target with

anti-apoptotic activity, PEG10 (paternally expressed 10), decreased in expression while the three miRNAs (miR-145, miR-432 and –miR-1972) capable of targeting it increased in expression.

There were a number of miRNAs that had multiple negatively correlated targets, not all of which were associated with the regulation of apoptosis. The miRNAs with the greatest numbers of negatively correlated targets included miR-134, miR-145 and miR-432 at 3h post EGF treatment and miR-494 and miR-146b-5p at 12 h (Supplementary Tables 1 and 2).

Discussion

Previously known miRNAs that regulate apoptosis found in our data.

It is known that miRNAs regulate individual components of multiple oncogenic pathways including the EGF pathway, that regulate various biological processes including apoptosis [39]. A number of miRNAs regulate the EGF signalling pathway, for instance MIR-145 can act as a tumour suppressor and down regulates several crucial oncogenes such as ki-RAS and v-myc, it is also down regulated in various types of cancer and has been shown to induce apoptosis [39, 40]. Our data show that MIR-145 is up regulated as a consequence of EGF treatment leading to apoptosis, which is consistent with previous results. We also found that miR-145 has a number of targets at 3h and 12h post EGF treatment in both gene-expression and proteome data (see Table 4, Table 6, Figure 2A, Supplementary Table 1).

Another miRNA with a know regulatory function of interest is miR-146b-5p, which plays an important role in regulating inflammation and when over expressed may also function to inhibit apoptosis. miR-146b-5p increases in abundance and has a number of targets (PLK2 (polo-like kinase 2), MYBL1 (v-myb myeloblastosis viral oncogene

homolog (avian)-like 1), NR2C2AP (nuclear receptor 2C2-associated protein), CTNNAL1 (catenin (cadherin-associated protein), alpha-like 1), DLK2 (delta-like 2 homolog (Drosophila), KIF24 (kinesin family member 24), MPHOSPH9 (M-phase phosphoprotein 9) and PER3 (period circadian clock 3) identified in our gene expression data that decrease in abundance at 3 and 12 h post EGF treatment. Of these, MYBL1 and NR2C2AP are anti-apoptotic, CTNNAL1 is pro-apoptotic and PLK2 has been reported as either pro or anti-apoptotic. So in our context, MIR-146b-5p may be acting to stimulate apoptosis.

Another miRNA of interest is miR-499-5p that has previously been shown to regulate FOXO4, which is a forkhead box O transcription factor, which can induce cell cycle arrest, DNA repair and apoptosis [41, 42]. Our results show miR-499-5p to be overexpressed at 3h post EGF treatment and then down regulated by 12h post EGF. This down regulation of miR-499-5p indicates that FOXO4 may be implicated in the induction of apoptosis in our system, or it may mean that miR-499 5p has other targets that can induce apoptosis that are relevant in our context, such as BTG1, GJB6 (gap junction protein, beta 6, 30kDa), TAGLN3 (transgelin 3), TNFSF15 (tumor necrosis factor (ligand) superfamily, member 15), APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G) and BHLHE41 (basic helix-loop-helix family, member e41)which had negatively correlated expression (Supplementary Table 1, Table 4A).

Perhaps of particular interest is miR-494, which has previously been shown to inhibit poly (ADP-ribose) polymerase PARP cleavage, the late event of apoptosis [43]. We observed a decrease in miR-494 abundance, which was correlated with increased expression of the following pro-apoptotic genes ID1, NCF2, TNFRSF9 (tumor necrosis factor receptor superfamily, member 9), and the pro-apoptotic protein PDIA3

(protein disulfide isomerase family A, member 3) (activates caspase3). Decrease in miR-494 expression was also correlated with increased expression of anti-apoptotic genes PLAUR (plasminogen activator, urokinase receptor), SERPINB3 (serpin peptidase inhibitor, clade B (ovalbumin), member 3) (both mRNA and protein) and SERPINB4 (serpin peptidase inhibitor, clade B (ovalbumin), member 3). Because ID1, NCF2, PDIA3, PLAUR and SERPINB3 are all known to be upregulated by the interferon/JAK/STAT pathway this suggests that miR-494 was either also downregulated by JAK/STAT as another regulatory loop within this context or was regulated independently to potentiate JAK/STAT activation. This is a novel hypothesis and is thus a good choice for further investigation with respect to the regulation of apoptosis in cancer cells.

Finally, miR-663 decreases in abundance at 3h and 12 h post EGF treatment and has previously been shown to mediate chemo-resistance of breast cancer cells by suppressing the apoptotic pathway [44]. Our data support this function of MIR-663, as its down regulation coincides with the up-regulation of the following pro-apoptotic genes (ID1, IL32, S100A9 (S100 calcium binding protein A9), CRABP2 (cellular retinoic acid binding protein 2) and TAGLN3). The regulation of ID1 and IL32 reinforces the prominence of the JAK/STAT signalling pathway in this context and miR-663 is therefore also a good candidate for further investigation in this context. .

miRNAs in our data that are novel candidates for the regulation of apoptosis.

In addition to the above miRNAs that we previously been shown to regulate apoptosis, we have identified another 11 miRNAs that are good candidates for a regulatory role in apoptosis. miR-146b-3p which is up regulated, has not previously been shown to induce apoptosis but it has a number of targets at 3h and 12h post EGF treatment,

some of which are known to regulate apoptosis (Figure 2, Table 4 and Supplementary Table 2).

Another novel miRNA in this context is miR-146b-5p, also up regulated, which is known to play an important role in regulating inflammation and when over expressed may also function to inhibit apoptosis. We have found that miR-146b-5p increases in abundance and has a number of targets (PLK2, MYBL1, NR2C2AP, CTNNAL1, DLK2, KIF24, MPHOSPH9 and PER3) identified in our gene expression data that decrease in abundance at 3h and 12h post EGF treatment. Of these, MYBL1 and NR2C2AP are anti-apoptotic, CTNNAL1 is pro-apoptotic and PLK2 have been reported as either pro or anti-apoptotic. So in our context, MIR-146b-5p may be acting to stimulate apoptosis.

Another novel miRNA of interest is miR-596, which decreased along with a correlated increase in its target, BTG1, known to promote apoptosis.

Another miRNA, miR-1231 that decreased in expression is also known to target BTG1 and in addition the pro-apoptotic gene TNFSF15, both of which increased in expression.

Another novel miRNA, miR-762 which was down regulated at 3h post EGF treatment targets the up-regulated apoptotic gene TNS4 (tensin 4) which is cleaved by Caspase 3 and is also known to bind to ACTB, reinforcing the role (see below) of ACTB and its regulators in apoptosis.

Regulation of the interferon/JAK/STAT pathway was also likely via miR-2861 which was down regulated and was correlated with an increase in the expression IRF1 at 3h post EGF and network NCF2 at 12 h post EGF. This strengthens the case for a miRNA regulatory loop that was down regulated in conjunction with activation of the interferon signalling pathways.

Another miRNA without previous connection to apoptosis is miR-3185 that was down regulated and correlated with up regulation of apoptotic genes ARHGEF2 (Rho/Rac guanine nucleotide exchange factor (GEF) 2) of the interferon pathway and DCUN1D3 (DCN1, defective in cullin neddylation 1, domain containing 3 (S. cerevisiae)) at 3h post EGF treatment. It was also down regulated at 12 h post EGF and correlated with increased expression of the apoptotic genes KCTD11 (potassium channel tetramerisation domain containing 11) and SORT1.

The miRNA miR-675 decreased in abundance and was correlated with an increase in the pro-apoptotic genes IL32 (regulated by the interferon pathway), KRT23 (keratin 23) and PI3 (peptidase inhibitor 3) at 12 h post EGF treatment.

Another down regulated miRNA, miR-602 was also correlated with the increased expression of the interferon pathway regulated ID1 at 12h post EGF treatment.

In addition, miR-638 also down regulated at 12h post EGF treatment, targets the pro-apoptotic gene SOD2 (superoxide dismutase 2, mitochondrial), which was up regulated.

Finally, miR-1972, which was up regulated, targets PEG10, which normally inhibits apoptosis (also targeted by miR-432 and miR-145) and the pro-apoptotic mutant TP53 at 12 hrs. Of the eleven novel candidate miRNAs that could regulate apoptosis, 9 were down regulated, leading to apparent de-repression of primarily pro-apoptotic targets and 2 were up regulated, leading to apparent suppression of anti-apoptotic targets.

Overlap between gene-expression and proteome miRNA targets

We have previously shown that there was little overlap between gene-expression and proteome data for A431 cells treated with EGF [18], with only 3 proteins matching

regulated transcripts, and none of those present in the correlation sub-networks. However, based on miRNAs, gene-expression and proteome networks may have more in common. We found 5 miRNAs (miR-29b-1star, miR-134, miR-145, miR-432 and miR-1909) that had targets in both the gene expression and proteomic sub-networks. As mentioned above, miR-145 is known to induce apoptosis and in the proteome data it targets ACTB as does miR-29b-1star (Figure 3, Table 6). This is of particular interest because it is known that actin cytoskeleton changes can trigger apoptosis [45]. miR-134 targets both MYB (gene expression) and BANF1 (proteome) but only MYB is known to regulate apoptosis (anti-apoptotic). However BANF1 is part of a sub-network that includes two proteins known to regulate apoptosis. miR-432 was the only miRNA that decreased at 3h and increased at 12h post EGF treatment and its target TCP1 also changed direction of gene expression at those times. TCP1, whilst not known to regulate apoptosis directly, is known to regulate actin and tubulin folding and is part of the same network as those two proteins (Figure 3). This also supports a role for miRNA regulation of cytoskeleton integrity and is consistent with promotion of apoptosis. The potential function of miR-1909 is unclear; even though it has targets at both 3h and 12h post EGF treatment in both the gene expression and proteome data sets, the direction of change in expression and annotation of the targets do not necessarily support a role for this miRNA as a regulator of apoptosis.

Conclusions

We have confirmed roles for some miRNAs in EGF signalling and apoptosis and implicated some miRNAs that previously had not been associated with EGF signalling or apoptosis. Our results suggest that miRNAs that target pro-apoptotic genes tend to decrease in abundance while their targets increase and that other

miRNAs that target anti-apoptotic genes increase in abundance while their targets decrease. Eight miRNAs (miR-494, miR-499-5p, miR-602, miR-663, miR-675, miR-1231, miR-2861 and miR-3185) that could normally target interferon pathway induced pro-apoptotic transcripts were found to be down regulated, leading us to conclude that miRNAs may provide a novel layer of regulation within the interferon pathway, specifically with respect to components downstream of JAK/STAT. Finally, we speculate that a pull/push mechanism exists to favour the expression of pro-apoptotic genes as a result of high dose EGF treatment.

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Supplementary materials

Supplementary Table 1: Differentially regulated interaction between miRNAs and genes in gene expression data in A431 cells after EGF treatment.

Supplementary Table 2: Differentially regulated interaction between miRNAs and genes in Proteomics data in A431 cells after EGF treatment.

Tables

Table 1: 37 MicroRNAs with significant changes to expression.

Table 2: Protein functional classes for gene expression and protein levels.

Table 3: Summary of miRNAs and their relationship to networks from gene expression and proteomic data.

Table 4: Differentially regulated interaction between miRNAs and genes where fold changes are negatively correlated in gene expression data.

Table 5: Negatively correlated interaction between miRNAs and genes expressed in Proteomics data.

Table 6: MicroRNAs Present In The Networks And Their Targets.

Figure Legends

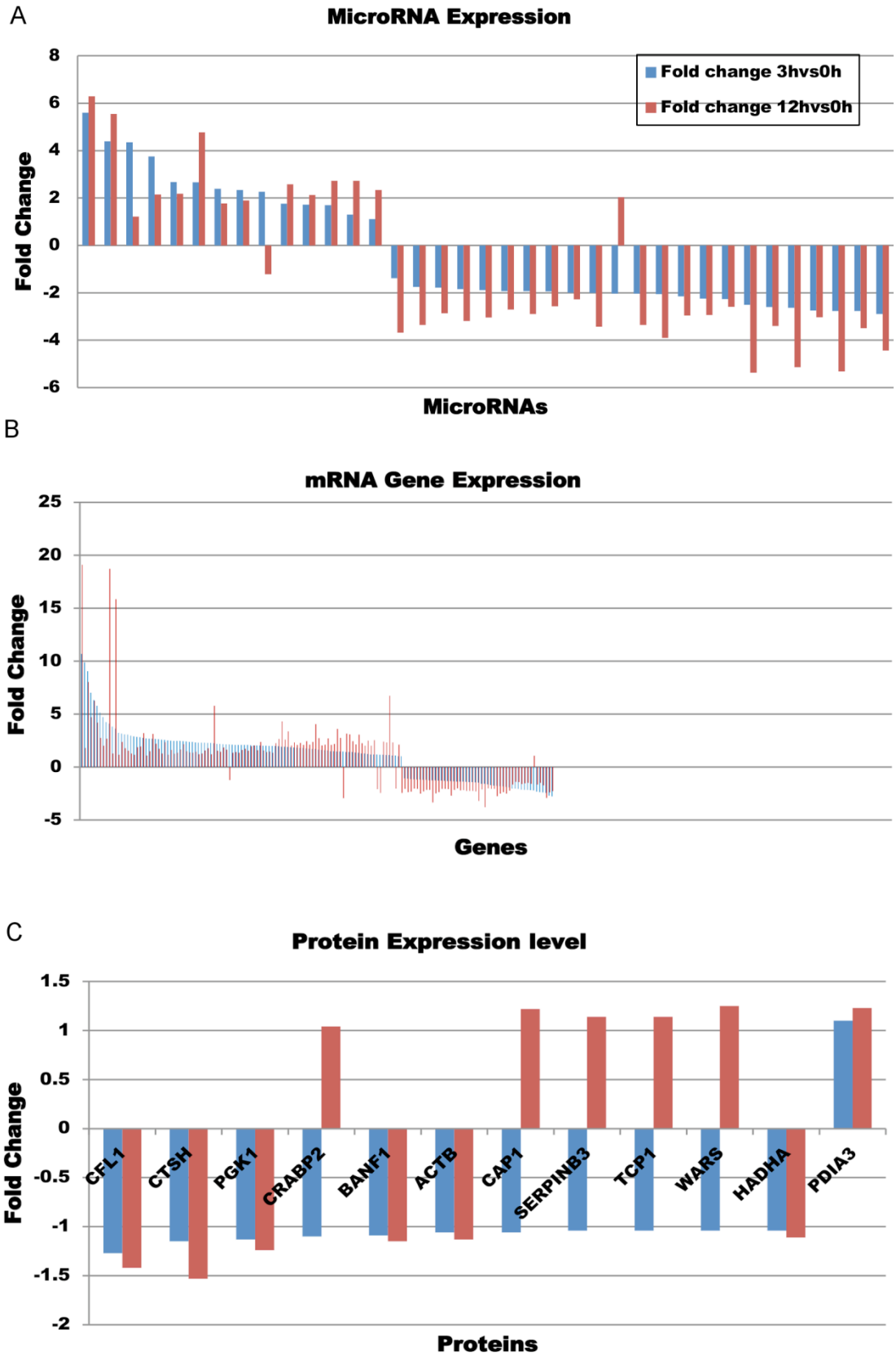
Figure 1. MicroRNAs, mRNA and protein expression at 3h vs 0h and 12h vs 0h fold change. Blue bars indicate 3h vs 0h fold change and red bars indicate 12h vs 0h fold change. (A) miRNA expression levels expressed as fold changes with respect to pre-treatment levels. (B) mRNA gene expression expressed as fold change with respect to pre-treatment levels. (C) Protein expression expressed as fold change with respect to pre-treatment levels.

Figure 2. Correlation gene expression networks based on single time point gene expression data. (A) pairwise gene expression data 0h-3h. (B) pairwise gene expression data 0h-12h. Anti-apoptotic genes coloured in pink, pro-apoptotic genes coloured in light blue, genes with no reported involvement in apoptosis coloured in

yellow. Octagonal shape indicates up-regulated genes. Squares for down-regulated genes. MicroRNAs negatively correlated with respect to target have red edges while MicroRNAs positively correlated with respect to target have blue edges.

Figure 3. Expression correlation network based on Proteomics data. There was no difference in the networks identified using pairwise proteomics data based on single time points from 3 or 12h after EGF treatment. Anti-apoptotic genes coloured in pink, pro-apoptotic genes coloured in light blue, genes with no reported involvement in apoptosis coloured in yellow. Octagonal shape indicates up-regulated genes. Squares for down-regulated genes. MicroRNAs negatively correlated with respect to target have red edges while MicroRNAs positively correlated with respect to target have blue edges. Diamonds indicate targets with isoforms that are both up and down regulated. Represented protein classes include: chaperone (TCP1 and CCT6A), cytoskeletal protein (EZR, DSTN, CAP1, TUBA1B and ACTB), isomerase (PPIA) and nucleic acid binding (XRCC6).

Figure 1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Fold change 12hvs0h
Fold Change
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2
0
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-4
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B
mRNA Gene Expression
Fold Change
25
20
15
10
5
0
-5
Genes
C
Protein Expression level
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0.5
0
-0.5
-1
-1.5
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CTSH
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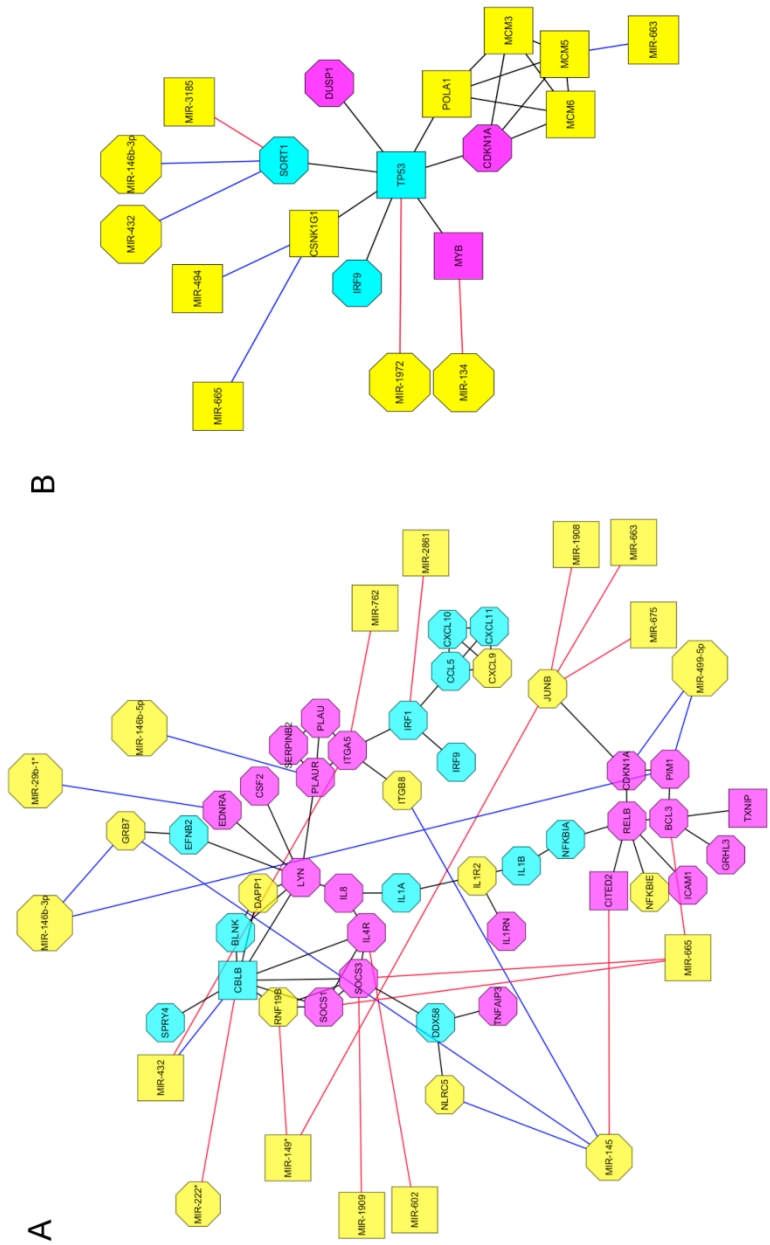
TCP1
WARS
HADHA
PDIA3
Proteins

Figure 2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MCM3
MCM5
MIR-663
DUSP1
POLA1
MIR-3185
MCM6
MIR-146b-3p
SORT1
TP53
CDKN1A
MIR-432
CSNK1G1
MYB
MIR-494
IRF9
MIR-1972
MIR-134
MIR-665
A
MIR-2861
MIR-1908
MIR-663
MIR-762
CXCL10
CXCL11
MIR-675
CCL5
CXCL9
JUNB
MIR-499-5p
MIR-146b-5p
PLAU
IRF1
SERPINB2
ITGA5
IRF9
MIR-29b-1*
PLAUR
CDKN1A
PIM1
CSF2
ITGB8
EDNRA
RELB
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TXNIP
GRB7
EFNB2
NFKBIA
GRHL3
IL1B
LYN
CITED2
ICAM1
MIR-146b-3p
DAPP1
IL8
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IL1R2
NFKBIE
BLNK
IL4R
IL1RN
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SOCS3
SPRY4
RNF19B
SOCS1
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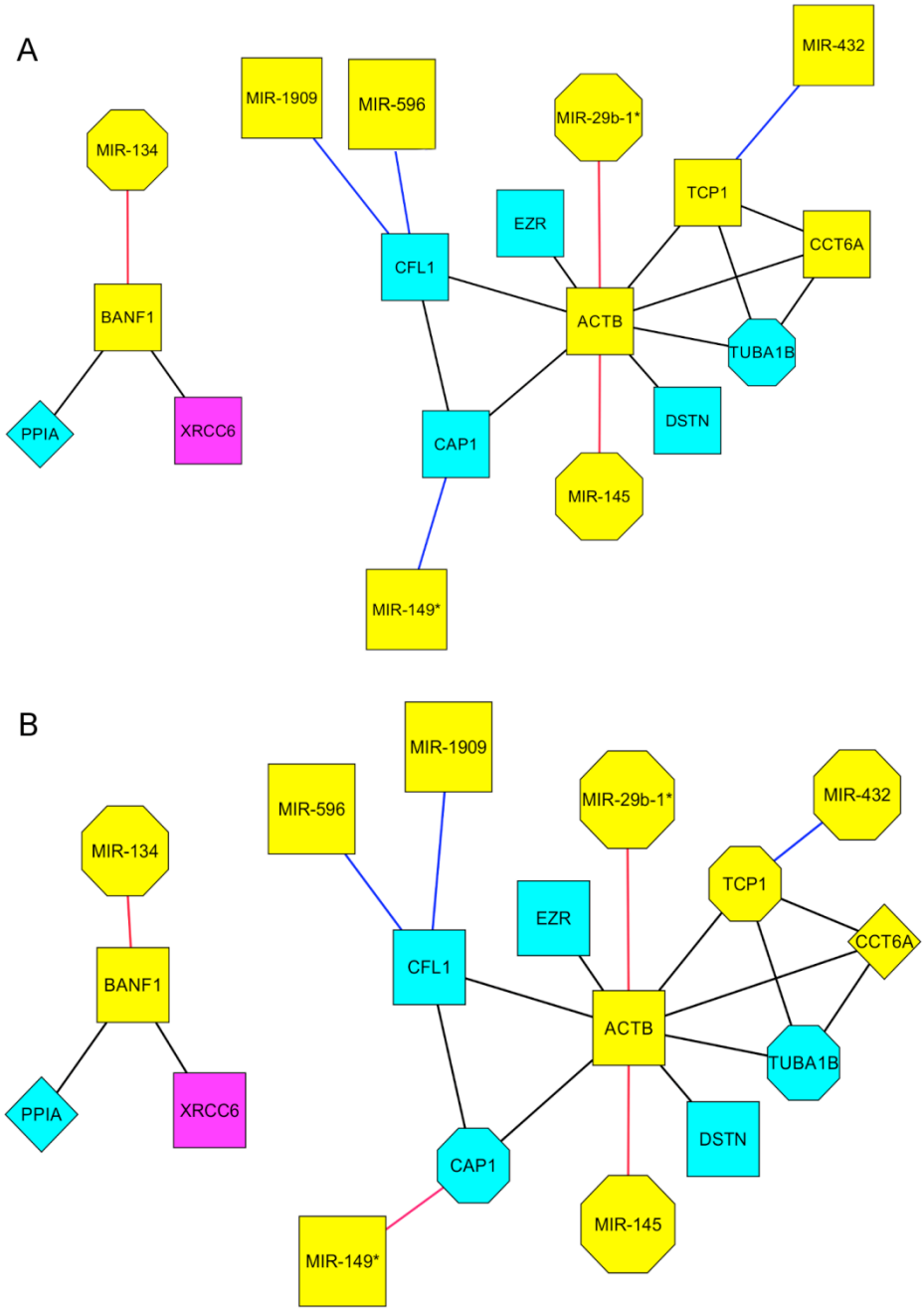
MIR-602

Figure 3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BANF1
PPIA
XRCC6
MIR-1909
MIR-596
CFL1
CAP1
MIR-149*
EZR
MIR-29b-1*
ACTB
MIR-145
TCP1
MIR-432
CCT6A
TUBA1B
DSTN
B
MIR-134
BANF1
PPIA
XRCC6
MIR-1909
MIR-596
CFL1
CAP1
MIR-149*
EZR
MIR-29b-1*
ACTB
MIR-145
TCP1
MIR-432
CCT6A
TUBA1B
DSTN

Table 1: 37 MicroRNAs with significant changes to expression.

MicroRNA	Apoptotic		Anti-apoptotic		Fold change 3h vs 0h	Fold change 12h vs 0h	P-Value	mRNA (M) / Protein (P) Target in networks
	3h vs 0h	12h vs 0h	3h vs 0h	12h vs 0h				
hsa-miR-1228-star_st	0	0	0	0	-2.06	-3.9	0.02	-
hsa-miR-146b-3p_st	3(1)*	5	1(1)*	1	5.6	6.29	0.02	M
hsa-miR-1908_st	0	0	0	0	-2	-3.43	0.02	-
hsa-miR-1909_st	1	1	1	0	-2.77	-5.32	0.02	M-P
hsa-miR-3180-3p_st	0	0	0	0	-2.51	-5.37	0.02	-
hsa-miR-4321_st	0	0	0	0	-1.75	-3.36	0.02	-
hsa-miR-596_st	2	2	0	1	-2.6	-3.4	0.02	P
hsa-miR-92a-1-star_st	0	0	1	0	4.39	5.55	0.02	-
hsa-miR-145_st	3	1	1	4	2.67	2.18	0.03	M-P
hsa-miR-29b-1-star_st	0	1	3	0	3.75	2.15	0.03	M-P
hsa-miR-432_st	1	2	1	2	-2.04	2.03	0.03	M-P
hsa-miR-663_st	2	5	0	1	-1.85	-3.19	0.03	M
hsa-miR-149-star_st	1	1	0	0	-1.93	-2.71	0.04	P
hsa-miR-602_st	0	1(1)*	1	2(1)*	-2.77	-3.49	0.04	M
hsa-miR-762_st	1	0	1	0	-2.04	-3.36	0.04	M
hsa-miR-134_st	2	4(1)*	1	4(1)*	2.39	1.77	0.05	M-P
hsa-miR-222-star_st	0	0	0	0	4.35	1.22	0.05	M
hsa-miR-665_st	0	0	3	2	-2.75	-3.03	0.05	M
hsa-miR-1231_st	2	0	0	0	-2.63	-5.14	0.06	-
hsa-miR-1469_st	0	0	0	0	-1.89	-3.04	0.06	-
hsa-miR-3185_st	2	2	0	0	-2.9	-4.44	0.07	M
hsa-miR-3195_st	0	0	0	0	-1.99	-2.28	0.07	-
hsa-miR-92b-star_st	0	0	0	0	-2.24	-2.94	0.07	-
hsa-miR-1972_st	2(1)*	2	1(1)*	1	1.3	2.73	0.08	M
hsa-miR-21-star_st	2	0	1	0	2.34	1.9	0.08	-
hsa-miR-23a-star_st	0	0	0	0	1.76	2.58	0.08	-
hsa-miR-27a-star_st	0	0	0	0	1.72	2.13	0.08	
hsa-miR-2861_st	1	1	0	0	-2.15	-2.96	0.08	M
hsa-miR-3178_st	0	0	0	0	-1.78	-2.86	0.08	-
hsa-miR-3179_st	0	0	0	0	1.11	2.34	0.08	-
hsa-miR-3181_st	0	0	0	0	-1.93	-2.9	0.08	-
hsa-miR-4304_st	0	0	0	0	2.66	4.77	0.08	-
hsa-miR-494_st	9	4(1)*	3	4(1)*	-1.38	-3.68	0.08	-
hsa-miR-499-5p_st	4	4	3	4	2.26	-1.22	0.08	M
hsa-miR-638_st	1	0	1	1	-1.94	-2.57	0.09	-
hsa-miR-146b-5p_st	2(1)*	1(1)*	3(1)*	5(1)*	1.7	2.73	0.1	M

hsa-miR-675_st	0	3	0	1	-2.27	-2.59	0.1	M

* () brackets indicate targets that have been annotated as both apoptotic and anti-apoptotic.

Table 2: Protein functional classes for gene expression and protein levels.

	Gene expression Protein classes		**Protein levels protein classes**	
protein-class	**3h vs 0h**	**12h vs 0h**	**3h vs 0h**	**12h vs 0h**
calcium-binding protein	√	√	-	-
cell adhesion molecule	√	√	-	-
cell junction protein	√	√	-	-
cytoskeletal protein	√	√	√	√
enzyme modulator	√	√	√	√
extracellular matrix protein	√	√	-	-
hydrolase	√	√	√	√
isomerase	√	-	√	√
kinase	√	√	√	√
ligase	√	√	√	√
lyase	√	-	√	√
nucleic acid binding	√	√	-	-
oxidoreductase	√	√	√	√
phosphatase	√	√	-	-
protease	√	√	√	√
receptor	√	√	-	-
signaling molecule	√	√	-	-
structural protein	√	√	-	-
transcription factor	√	√	-	-
transfer/carrier protein	√	-	√	√
transferase	√	√	√	√
transmembrane receptor regulatory/adaptor protein	√	-	-	-
transporter	√	√	-	-
defense/immunity protein	-	√	-	-
membrane traffic protein	-	√	-	-
chaperone	-	-	√	√

Table 3: Summary of miRNAs and their relationship to networks from gene expression and proteomic data.

microRNA	Gene expression		Protein level	
[1] ID	3hvs0h fold change[2]	12hvs0h fold change[2]	3hvs0h fold change[3]	12hvs0h fold change[3]
hsa-miR-134_st	–	√	√	√
hsa-miR-145_st	√	–	√	√
hsa-miR-146b-3p_st	√	√	–	–
hsa-miR-146b-5p_st	√	–	–	–
hsa-miR-149-star_st	√	–	√	√
hsa-miR-1908_st	√	–	–	–
hsa-miR-1909_st	√	–	√	√
hsa-miR-1972_st	–	√	–	–
hsa-miR-222-star_st	√	–	–	–
hsa-miR-2861_st	√	–	–	–
hsa-miR-29b-1-star_st	√	–	√	√
hsa-miR-3185_st	–	√	–	–
hsa-miR-432_st	√	√	√	√
hsa-miR-494_st	–	√	–	–
hsa-miR-499-5p_st	√	–	–	–
hsa-miR-596_st	–	–	√	√
hsa-miR-602_st	√	–	–	–
hsa-miR-663_st	√	√	–	–
hsa-miR-665_st	√	√	–	–
hsa-miR-675_st	√	–	–	–
hsa-miR-762_st	√	–	–	–

[1] Significant fold change with adjusted p value with FDR 0.1 as can be seen in supplementary Table 1.
[2] Significant fold change with p value < 0.05 as can be seen in supplementary Table 2.
[3] Significant fold change based on DeCyder software one-way ANOVA analysis of p value < 0.05 as can be seen in supplementary Table 3.

Table 4: Differentially regulated interaction between miRNAs and genes where fold changes are negatively correlated, in gene expression data.

A- MicroRNA gene expression with their apoptotic or anti-apoptotic target mRNA gene expression at 3h, sorted by target.

miRNA ID	miRNA fold change 3h vs 0h	Target gene	mRNA fold Change 3h vs 0h	Apoptotic or anti apoptotic Function
hsa-miR-3185	-2.9	ARHGEF2	2.42	Apoptotic
hsa-miR-665	-2.75	BCL3	3.81	Anti-apoptotic
hsa-miR-499-5p	2.26	BHLHE41	-2.37	Anti-apoptotic
hsa-miR-596	-2.6	BTG1	2.09	Apoptotic
hsa-miR-1231	-2.63	BTG1	2.09	Apoptotic
hsa-miR-145	2.67	CITED2	-2.21	Anti-apoptotic
hsa-miR-3185	-2.9	DCUN1D3	3.08	Apoptotic
hsa-miR-145	2.67	FBXO32	-2.77	Apoptotic
hsa-miR-145	2.67	HOXA5	-2.04	Apoptotic
hsa-miR-602	-2.77	IL4R	2.02	Anti-apoptotic
hsa-miR-2861	-2.15	IRF1	2.95	Apoptotic
hsa-miR-762	-2.04	ITGA5	2.46	Anti-apoptotic
hsa-miR-432	-2.04	ITGA5	2.46	Anti-apoptotic
hsa-miR-146b-3p	5.6	PHF17	-2.07	Apoptotic/ Anti-apoptotic
hsa-miR-665	-2.75	SOCS1	2.64	Anti-apoptotic
hsa-miR-665	-2.75	SOCS3	3.05	Anti-apoptotic
hsa-miR-1909	-2.77	SOCS3	3.05	Anti-apoptotic
hsa-miR-1231	-2.63	TNFSF15	2	Apoptotic
hsa-miR-762	-2.04	TNS4	2.14	Apoptotic
hsa-miR-21*	2.34	WEE1	-2.39	Anti-apoptotic

B- MicroRNA gene expression with their apoptotic or anti-apoptotic target mRNA gene expression at 12h, sorted by target.

miRNA ID	miRNA fold change 12h vs 0h	Target gene	mRNA fold Change 12h vs 0h	Apoptotic or anti apoptotic Function
hsa-miR-663	-3.19	CNFN	6.73	Anti-apoptotic
hsa-miR-146b-5p	2.73	CTNNAL1	-2.32	Apoptotic
hsa-miR-145	2.18	FBXO32	-2.28	Apoptotic
hsa-miR-663	-3.19	ID1	2.65	Apoptotic/ Anti-apoptotic
hsa-miR-602	-3.49	ID1	2.65	Apoptotic/ Anti-apoptotic
hsa-miR-494	-3.68	ID1	2.65	Apoptotic/ Anti-apoptotic
hsa-miR-675	-2.59	IL32	2.03	Apoptotic
hsa-miR-663	-3.19	IL32	2.03	Apoptotic
hsa-miR-3185	-4.44	KCTD11	2.01	Apoptotic
hsa-miR-675	-2.59	KRT23	3.36	Apoptotic
hsa-miR-145	2.18	MPP1	-2.7	Anti-apoptotic
hsa-miR-675	-2.59	MUC1	2.03	Anti-apoptotic
hsa-miR-665	-3.03	MUC1	2.03	Anti-apoptotic
hsa-miR-146b-5p	2.73	MYBL1	-2.74	Anti-apoptotic
hsa-miR-494	-3.68	NCF2	4.3	Apoptotic
hsa-miR-2861	-2.96	NCF2	4.3	Apoptotic
hsa-miR-146b-5p	2.73	NR2C2AP	-2.04	Anti-apoptotic
hsa-miR-29b-1*	2.15	PBX1	-2.48	Apoptotic

hsa-miR-146b-3p	6.29	PBX1	-2.48	Apoptotic
hsa-miR-602	-3.49	PDZK1IP1	3.61	Anti-apoptotic
hsa-miR-432	2.03	PEG10	-3.33	Anti-apoptotic
hsa-miR-1972	2.73	PEG10	-3.33	Anti-apoptotic
hsa-miR-145	2.18	PEG10	-3.33	Anti-apoptotic
hsa-miR-675	-2.59	PI3	4.06	Apoptotic
hsa-miR-494	-3.68	PLAUR	2.76	Anti-apoptotic
hsa-miR-146b-5p	2.73	PLK2	-2.92	Apoptotic/Anti-apoptotic
hsa-miR-665	-3.03	S100A2	2.36	Anti-apoptotic
hsa-miR-663	-3.19	S100A9	3.07	Apoptotic
hsa-miR-494	-3.68	SERPINB3	18.71	Anti-apoptotic
hsa-miR-494	-3.68	SERPINB4	15.85	Anti-apoptotic
hsa-miR-596	-3.4	SLPI	2.58	Anti-apoptotic
hsa-miR-638	-2.57	SOD2	2.34	Anti-apoptotic
hsa-miR-3185	-4.44	SORT1	2.14	Apoptotic
hsa-miR-663	-3.19	TAGLN3	2.38	Apoptotic
hsa-miR-494	-3.68	TNFRSF9	2.16	Apoptotic
hsa-miR-1972	2.73	TP53	-2.5	Apoptotic

Table 5: Negatively correlated interaction between miRNAs and genes expressed in Proteomics data.

A- Protein expression and miRNA expression at 3h.

miRNA ID	miRNA fold change 3h vs 0h	Target ID	Combined IonScore	Protein fold change3h vs 0h	Apoptotic Function
hsa-miR-134	2.39	CRABP2	249	-1.1	Apoptotic

B- Protein expression and miRNA expression at 12h.

miRNA ID	miRNA fold change 12h vs 0h	Target ID	Combined IonScore	Protein fold change 12h vs 0h	Apoptotic Function
hsa-miR-149*	-2.71	CAP1	778	1.22	Apoptotic
hsa-miR-663	-3.19	CRABP2	249	1.04	Apoptotic
hsa-miR-494	-3.68	PDIA3	1268	1.23	Apoptotic
hsa-miR-494	-3.68	SERPINB3	245	1.14	Anti-apoptotic

Table 6: MicroRNAs Present In The Networks And Their Targets.

A- MicroRNA expression and target mRNA gene expression.

miRNA ID	miRNA fold change		Target gene	mRNA fold Change		Apoptotic or anti-apoptotic Function
	3h vs 0h	12h vs 0h		3h vs 0h	12h vs 0h	
hsa-miR-29b-1*	3.75	2.15	CFLAR	2.46	1.34	Anti-apoptotic
hsa-miR-145	2.67	2.18	CITED2	-2.21	1.06	Anti-apoptotic
hsa-miR-29b-1*	3.75	2.15	EDNRA	2.16	1.85	Anti-apoptotic
hsa-miR-432	-2.04	2.03	ITGA5	2.46	1.66	Anti-apoptotic
hsa-miR-145	2.67	2.18	MPP1	-1.33	-2.7	Anti-apoptotic
hsa-miR-145	2.67	2.18	MUC1	1.88	2.03	Anti-apoptotic
hsa-miR-134	2.39	1.77	MYB	-1.91	-2.23	Anti-apoptotic
hsa-miR-29b-1*	3.75	2.15	NUAK2	2.59	1.28	Anti-apoptotic
hsa-miR-145	2.67	2.18	PDZK1IP1	1.48	3.61	Anti-apoptotic
hsa-miR-134	2.39	1.77	PDZK1IP1	1.48	3.61	Anti-apoptotic
hsa-miR-145	2.67	2.18	PEG10	-1.26	-3.33	Anti-apoptotic
hsa-miR-432	-2.04	2.03	PEG10	-1.26	-3.33	Anti-apoptotic
hsa-miR-432	-2.04	2.03	S100A2	1.71	2.36	Anti-apoptotic
hsa-miR-1909	-2.77	-5.32	SOCS3	3.05	1.55	Anti-apoptotic
hsa-miR-134	2.39	1.77	SOD2	2.55	2.34	Anti-apoptotic
hsa-miR-145	2.67	2.18	BTG1	2.09	1.43	Apoptotic
hsa-miR-145	2.67	2.18	FBXO32	-2.77	-2.28	Apoptotic
hsa-miR-145	2.67	2.18	HOXA5	-2.04	-1.65	Apoptotic
hsa-miR-432	-2.04	2.03	HOXA5	-2.04	-1.65	Apoptotic
hsa-miR-134	2.39	1.77	ID1	1.96	2.65	Apoptotic
hsa-miR-134	2.39	1.77	IL13RA2	1.19	2.02	Apoptotic
hsa-miR-29b-1*	3.75	2.15	PBX1	-1.26	-2.48	Apoptotic
hsa-miR-432	-2.04	2.03	S100A9	1.42	3.07	Apoptotic
hsa-miR-432	-2.04	2.03	SORT1	1.38	2.14	Apoptotic
hsa-miR-134	2.39	1.77	TAGLN3	3.15	2.38	Apoptotic
hsa-miR-432	-2.04	2.03	APOBEC3G	2.09	1.36	Other function
hsa-miR-432	-2.04	2.03	ARL5B	2.36	1.34	Other function
hsa-miR-145	2.67	2.18	ARL5B	2.36	1.34	Other function
hsa-miR-432	-2.04	2.03	ARSI	-1.42	-2.24	other function
hsa-miR-432	-2.04	2.03	BCAM	1.01	-2.44	other function
hsa-miR-432	-2.04	2.03	C1orf74	2.49	1.6	Other function
hsa-miR-432	-2.04	2.03	CBLB	-2.15	-1.54	Other function
hsa-miR-145	2.67	2.18	CCL20	2.3	1.21	Other function
hsa-miR-432	-2.04	2.03	DHRS9	1.64	2.73	other function
hsa-miR-134	2.39	1.77	DHRS9	1.64	2.73	other function
hsa-miR-145	2.67	2.18	FLNB	1.5	2.08	other function
hsa-miR-432	-2.04	2.03	GK	1.19	2.53	other function
hsa-miR-145	2.67	2.18	GRB7	2.27	1.77	Other function
hsa-miR-29b-1*	3.75	2.15	GRHL1	2.75	3.19	Other function
hsa-miR-145	2.67	2.18	HSD17B2	1.16	2.4	other function
hsa-miR-145	2.67	2.18	ITGB8	2.33	1.45	Other function
hsa-miR-134	2.39	1.77	ITGB8	2.33	1.45	Other function
hsa-miR-145	2.67	2.18	JHDM1D	7.01	4.71	Other function
hsa-miR-432	-2.04	2.03	JHDM1D	7.01	4.71	Other function

hsa-miR-432	-2.04	2.03	MACC1	2.9	1.17	Other function
hsa-miR-29b-1*	3.75	2.15	MACC1	2.9	1.17	Other function
hsa-miR-432	-2.04	2.03	MAML2	-1.38	-2.19	other function
hsa-miR-145	2.67	2.18	MFSD2A	2.66	2.2	Other function
hsa-miR-29b-1*	3.75	2.15	MPPED2	-1.59	-3.8	other function
hsa-miR-134	2.39	1.77	MRM1	-1.63	-2.02	other function
hsa-miR-432	-2.04	2.03	MXRA5	-1.25	-2.16	other function
hsa-miR-145	2.67	2.18	NAV3	1.58	2.1	other function
hsa-miR-145	2.67	2.18	NFIB	-1.19	-2.07	other function
hsa-miR-1909	-2.77	-5.32	NFIB	-1.19	-2.07	other function
hsa-miR-145	2.67	2.18	NLRC5	2.52	1.17	Other function
hsa-miR-432	-2.04	2.03	NRM	-1.2	-2.3	other function
hsa-miR-145	2.67	2.18	NTN4	-1.32	-2.08	other function
hsa-miR-145	2.67	2.18	PLCE1	-1.22	-2.15	other function
hsa-miR-432	-2.04	2.03	PRSS22	2.82	1.95	Other function
hsa-miR-1909	-2.77	-5.32	RAB43	2.67	1.5	Other function
hsa-miR-134	2.39	1.77	RHCG	1.1	2.32	other function
hsa-miR-134	2.39	1.77	SDCBP2	1.47	2.75	other function
hsa-miR-1909	-2.77	-5.32	SEMA7A	3.23	1.16	Other function
hsa-miR-134	2.39	1.77	SPRR2A	6.33	6.25	Other function
hsa-miR-145	2.67	2.18	SQRDL	1.56	2.69	other function
hsa-miR-134	2.39	1.77	SQRDL	1.56	2.69	other function
hsa-miR-145	2.67	2.18	SRGAP1	-2.19	-1.58	Other function
hsa-miR-432	-2.04	2.03	THNSL1	-2.31	-1.67	Other function

B- MicroRNA expression and Protein (target) expression.

miRNA ID	**miRNA fold change**		**Target ID**	**Combined IonScore**	**Protein fold change**		**Apoptotic or anti-apoptotic Function**
	3h vs 0h	**12h vs 0h**			**3h vs 0h**	**12h vs 0h**	
hsa-miR-1909	-2.77	-5.32	CFL1	984	-1.27	-1.42	Apoptotic
hsa-miR-134	2.39	1.77	CRABP2	249	-1.1	1.04	Apoptotic
hsa-miR-145	2.67	2.18	ACTB	357	-1.06	-1.13	Other function
hsa-miR-29b-1*	3.75	2.15	ACTB	357	-1.06	-1.13	Other function
hsa-miR-134	2.39	1.77	BANF1	158	-1.09	-1.15	Other function
hsa-miR-432	-2.04	2.03	TCP1	2763	-1.04	1.14	Other function

CHAPTER FOUR

A comprehensive Catalogue of Differentially Expressed Genes and Proteins From Epidermal growth factor (EGF) induced Apoptosis in A431 cells

A comprehensive Catalogue of Differentially Expressed Genes and Proteins From Epidermal growth factor (EGF) induced Apoptosis in A431 cells

Abstract

The deregulation of HER1 and HER2 receptors may cause the development of a variety of cancers including breast cancer and lung cancer. Recent studies have revealed that the recognition of deregulated molecular pathways in cancer may be more useful than recognition of individual genes. Since the same pathway can be deregulated by various subsets of genes, it is important to study pathways as a whole, rather than focus on individual genes. Because of the crucial importance of the EGFR pathway in apoptosis induced by Epidermal growth factor (EGF) in A431 (as a model to study breast cancer), we used bioinformatics databases and *in silico* analysis tools to create a comprehensive catalogue of genes inducing apoptosis in A431 cell after EGF treatment. This catalogue includes gene clustering analysis, gene ontology (GO) terms, protein domain and functional classification.

Introduction

The Epidermal Growth Factor Receptor (EGFR/HER1) pathway is a crucial signaling pathway involved in different biological processes such as cell proliferation, development, differentiation [1], cell survival and cell migration [2]. EGFR signaling begins with the binding of growth factors, such as EGF, to trans-membrane receptors on the cell surface (HER1, HER12, HER3, and HER4). As a result of ligand binding these receptors will homo- or hetero-dimerize, followed by auto-phosphorylation. The phosphorylation of tyrosine residues on the cytoplasmic domain creates docking sites for intracellular effector and adaptor proteins that will be released in active form to stimulate signal transduction networks within the cells [3]. HER1 activation regulates several of the main cellular signaling mechanisms including the Phospholipase Cγ pathway (intracellular calcium release), STAT pathway (gene expression) and SRC pathway (cell proliferation, cell migration, cell adhesion, angiogenesis and immune function), RAS/RAF/MAPK pathway (cell proliferation and survival), PI3-K/AKT pathway (cell growth, apoptosis resistance, invasion and migration)[4]. However, the activation of HER1 by EGF also induces apoptosis in cell lines that over-express HER 1 such as A431 cells [5-11]. The over

expression of HER1 and HER2 receptors have been observed in various types of cancer including bladder and lung cancer, colon carcinoma [1, 12], and breast cancer [13].

Here we have used a variety of approaches including microarray and proteomics platforms to identify the downstream components that induce apoptosis in treated A431 cells was used as a model to study breast cancer. Using the data that we obtained from microarray and proteomics platforms, we created a comprehensive catalogue of differentially expressed genes and proteins involved in inducing apoptosis in A431 cells after EGF treatment in order to clarify how apoptosis occurs in these cells.

Gene Clustering and Classification

Microarray technology has enabled the quantitative measurement of thousands of genes concurrently. This approach allows whole genomes to be scanned, generating thousands of data points from each microarray experiment [14]. We used this platform to identify genes differentially expressed in treated cells compared to untreated cells. With the proteiomics platform, two-dimensional fluorescence difference in gel electrophoresis (2D-DIGE) and Electrospray Ionisation/Liquid Chromatography tandem Mass Spectrometry (ESI-LC-MS/MS) were used to identify differentially expressed proteins in treated cells compared to untreated cells. To reveal the various functions of the genes and proteins identified in treated cells, the gene expression and protein profiles can be analysed to group the genes/proteins into clusters based on similarity in their patterns of expression. These co-expression clusters may be interpreted as functional groupings for genes/proteins; each cluster containing genes/proteins likely required for a common function.

Gene Ontology (GO)

Gene Ontology is used to analyze results from microarray and proteomics platforms to determine which biological process and molecular functions are significantly over or under-represented in a group of genes/proteins in treated cells. Gene Ontology (GO) is a collection of controlled vocabularies that illustrate the biological classification of a gene product [15]. GO provides both ontologies and annotations for three distinct areas of cell biology including Biological Process

(BP), Cellular Component (CC), and Molecular function (MF)[16]. The interpretation of the result of such an experiments requires statistical testing for over and under representation of gene function categories [17].

Go enrichment analysis was performed using DAVID Bioinformatics Resources 6.7 [18]. GO term output was submitted to the REVIGO web server. This software is capable of summarizing long lists of GO terms by finding a representative subset of the terms using a clustering algorithm based on semantic similarity measures [19].

Determining Protein Domain and protein families

The PFAM database was used to determine if a set of genes/proteins was related to a particular protein family. The PFAM database is a large collection of protein families and domains, with a range of well-established uses including genome annotation [20]. In general proteins consist of several functional subunits, which combine and act as a protein complex. The interaction of these subunits provides functional and /or structural diversity [21]. A number of processes in cells are controlled by protein-protein interactions. It has been recognized that these interactions are mediated by the binding of polypeptide segments to folded domains [22]. The families of modular domains differ in their three dimensional folding and ligand binding specificity. Therefore, the members of the same families share the same fold and specificity [23].

Functional Classification

PANTHER has been utilized to obtain functional classification information that can assist finding biological pattern in the data obtained from microarray and mass spectrometry data [24]. PANTHER (Protein ANalysis THrough Evolutionary Relationships) is a freely available, comprehensive software system for relating gene/protein sequence evolution, specific gene/protein functions and biological roles [25, 26]. PANTHER consists of three types of gene attribute including subfamily membership, protein class and gene function [27]. Therefore, PANTHER software was used to identify pathways and some of the proteins classes including kinase, receptor, transcription factors (T.Fs), and calcium binding protein from gene and protein data that were obtained from microarray and proteomics experiments respectively in order to

clarify which kind of pathways and protein class(s) were involved in the induction of apoptosis.

Results

Differential Gene Expression between treatments and controls in A431 cells

Using genome-wide measurements of gene expression levels during the treatment with EGF, the functional associations of various genes were clustered based on their similarities in gene expression. From the gene expression data, 217, 251, 213, and 207 genes with significantly changed expression were selected at 3 hours, 12 hours, 16 hours, and 24 hours after EGF treatment respectively. These genes were differentially expressed in comparison with control based on fold change (≥ 2 and ≤ -2) and P-value <0.05. We constructed gene clusters from 425 genes that were up or down-regulated (≥ 2 and ≤ -2) respectively from all time points provided they were significant ($P < 0.05$) (Supplementary Table S1). Gene clustering for these genes can be seen in figure 1. The co- expression of clusters can be inferred as biological functional grouping for the genes.

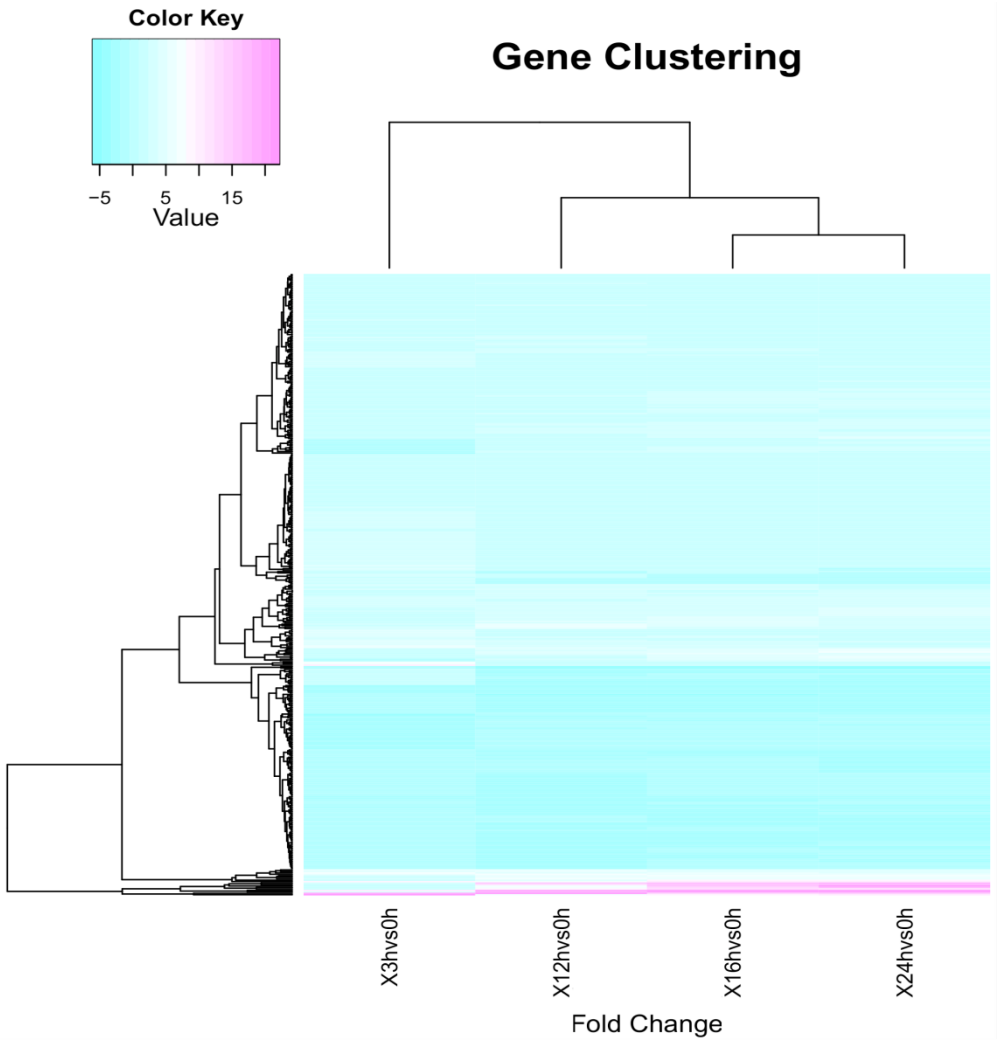

Figure 1: Hierarchical clustering of the A431 time course treatment samples based on expression of 425 selected genes. Each column represents a fold change and each row represents a gene (Gene symbol identifier were eliminated because there were to many, making it hard to see them in the figure). Relative levels of gene expression are depicted with a color scale where pink represents the highest level of expression and light blue represents the lowest level. Clustering was performed using a hierarchical clustering algorithm with Euclidean distance and average linkage clustering method.

Differential protein level between treatments and controls in A431 cells

Two-dimensional fluorescence difference in gel electrophoresis (2D-DIGE) and Electrospray Ionisation/ Liquid Chromatography tandem Mass Spectrometry (ESI-LC-MS/MS) were used to measure changes in protein level after the treatment of A431 cells with EGF. The functional clustering of various proteins was based on their similar protein expression patterns. Using

proteomics data, 63 spots (50 proteins, multiple proteins found in some spots) were selected based on the changing of protein levels during the time point experiment with P-value <0.05 using DeCyder software. Using LC-MS/MS, 832 proteins from these spots were identified (Supplementary Table S2). Then, 50 out of 832 proteins were chosen based on their highest combined ion score. Keratin was eliminated from each spot to avoid gel contamination during the electrophoresis process (Supplementary Table S3). Protein clustering for these proteins can be seen in figure 2.

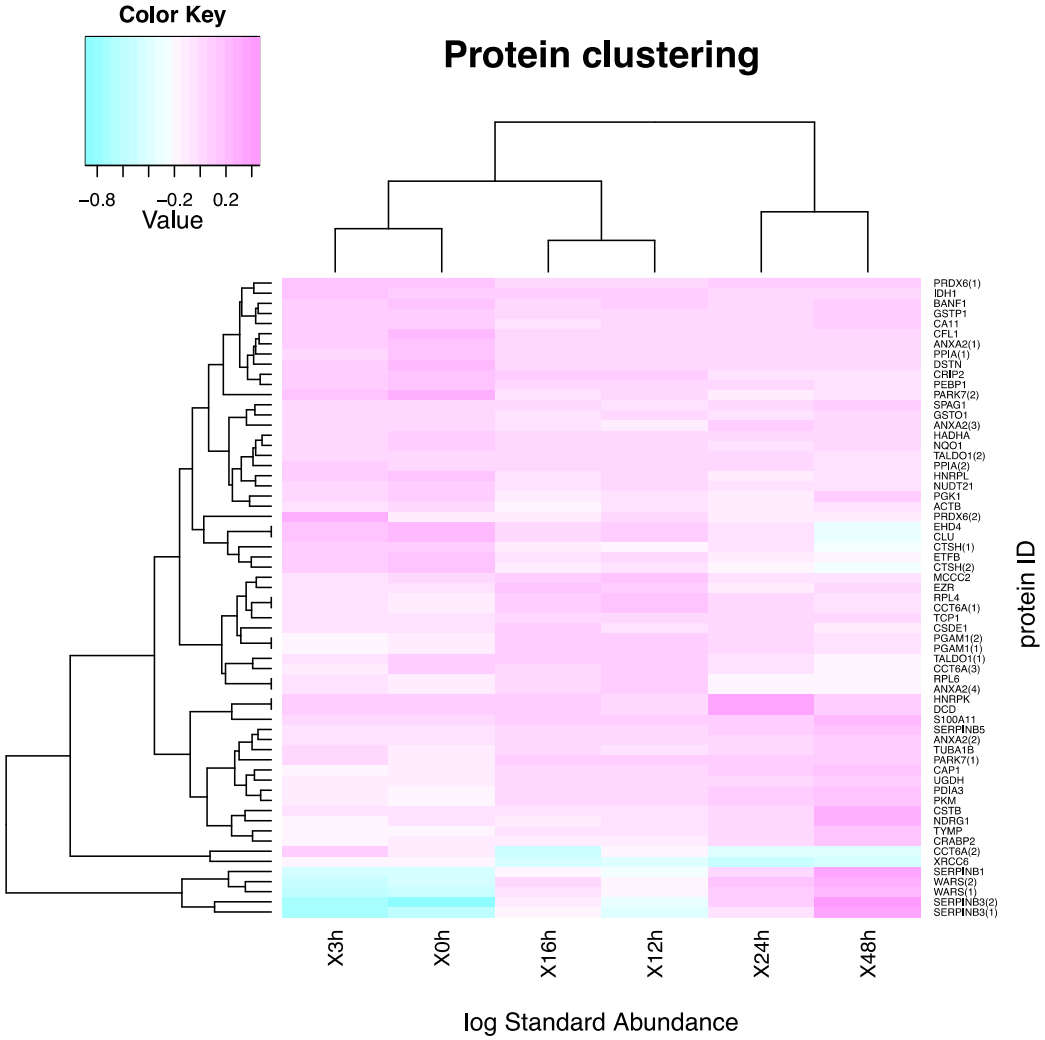

Figure 2: Hierarchical clustering of the A431 time course treatment samples based on expression of 50 selected proteins. Each column represents a log standard abundance and each row represents a protein. Relative levels of protein level are depicted with a color scale where pink represents the highest level of expression and light blue represents the lowest level. Clustering was performed using hierarchical

clustering algorithm with Euclidean distance and average linkage clustering method.

GO terms associated with differentially expressed genes

GO enrichment analysis was performed using DAVID Bioinformatics Resources 6.7 for up and down-regulated gene expression at each time point to determine which biological processes and molecular functions were significantly over or under represented in a group of genes in treated A431 cells. The number of GO terms that were significantly over and under represented after EGF treatment, can be seen in Table 1. These GO terms were based on Fisher's exact test using the EASE modification ($P < 0.05$) and multiple testing corrections was performed using the Benjamini-Hochberg FDR method Supplementary Table S4 and Table S5. Among different GO terms of up-regulated genes, several enriched terms in the biological process and molecular function categories related to the molecular mechanisms associated with cytokine activity, regulation of programmed cell death, taxis, chemotaxis, inflammatory response, keratinocyte differentiation and others were observed (Table 2). While GO terms associated with down-regulated genes were enriched for various terms related to transcription factor activity, DNA metabolic process, nucleosome assembly, nuclear division and others (Table 3). These results suggest that EGF treated A431 cells differentially expressed genes involved in immune response, regulation of programmed cell death and inflammation. This supported our finding that treatment of A431 cells with a high concentration of EGF may be capable of inducing apoptosis via apoptotic genes such as TP53 and SERPINB1[28].

Table 1: Number of GO terms in each time point post EGF treatment.

GO terms	3h vs 0h	12h vs 0h	16h vs 0h	24h vs 0h
Up regulated genes	167	44	51	42
Down regulated genes	15	17	14	21

Table 2: Statistically significant GO terms (biological process and molecular function categories) derived from up-regulated genes at 3h, 12h, 16h and 24.

3h (Treatment) vs 0h (control)		
Category	**Term**	**P-value**
GOTERM_MF_4	GO:0005125:cytokine activity	2.04E-12
GOTERM_BP_4	GO:0043067:regulation of programmed cell death	5.25E-09
GOTERM_BP_4	GO:0010941:regulation of cell death	5.69E-09
GOTERM_BP_4	GO:0042127:regulation of cell proliferation	1.16E-08
GOTERM_BP_4	GO:0048523:negative regulation of cellular process	1.42E-08
GOTERM_BP_4	GO:0042330:taxis	6.22E-08
GOTERM_BP_4	GO:0006935:chemotaxis	6.22E-08
GOTERM_BP_4	GO:0043066:negative regulation of apoptosis	7.81E-08
GOTERM_BP_4	GO:0043069:negative regulation of programmed cell death	9.56E-08
GOTERM_BP_4	GO:0060548:negative regulation of cell death	9.96E-08
12h (Treatment) vs 0h (control)		
Category	**Term**	**P-value**
GOTERM_BP_4	GO:0042330:taxis	5.96E-06
GOTERM_BP_4	GO:0006935:chemotaxis	5.96E-06
GOTERM_MF_4	GO:0005125:cytokine activity	7.01E-06
GOTERM_BP_4	GO:0006954:inflammatory response	6.54E-05
GOTERM_BP_4	GO:0030855:epithelial cell differentiation	1.24E-04
GOTERM_BP_4	GO:0008544:epidermis development	1.25E-04
GOTERM_BP_4	GO:0033273:response to vitamin	1.94E-04
GOTERM_BP_4	GO:0030216:keratinocyte differentiation	1.94E-04
GOTERM_BP_4	GO:0007398:ectoderm development	2.14E-04
GOTERM_BP_4	GO:0009913:epidermal cell differentiation	2.92E-04
16h (Treatment) vs 0h (control)		
Category	**Term**	**P-value**
GOTERM_BP_4	GO:0030216:keratinocyte differentiation	6.08E-08
GOTERM_BP_4	GO:0007398:ectoderm development	7.95E-08
GOTERM_BP_4	GO:0009913:epidermal cell differentiation	1.22E-07
GOTERM_BP_4	GO:0030855:epithelial cell differentiation	1.73E-07
GOTERM_BP_4	GO:0008544:epidermis development	3.11E-07
GOTERM_BP_4	GO:0048513:organ development	6.11E-07
GOTERM_BP_4	GO:0006935:chemotaxis	7.35E-07
GOTERM_BP_4	GO:0042330:taxis	7.35E-07
GOTERM_BP_4	GO:0006954:inflammatory response	1.39E-05
GOTERM_BP_4	GO:0060429:epithelium development	1.68E-05
24h (Treatment) vs 0h (control)		
Category	**Term**	**P-value**
GOTERM_BP_4	GO:0030216:keratinocyte differentiation	3.32E-08
GOTERM_BP_4	GO:0009913:epidermal cell differentiation	6.66E-08
GOTERM_BP_4	GO:0030855:epithelial cell differentiation	8.31E-08
GOTERM_BP_4	GO:0008544:epidermis development	1.41E-07
GOTERM_BP_4	GO:0007398:ectoderm development	3.11E-07
GOTERM_BP_4	GO:0042330:taxis	3.40E-06
GOTERM_BP_4	GO:0006935:chemotaxis	3.40E-06
GOTERM_BP_4	GO:0006954:inflammatory response	6.23E-06
GOTERM_BP_4	GO:0048513:organ development	8.21E-06
GOTERM_BP_4	GO:0060429:epithelium development	8.46E-06

Table 3: Statistically significant GO terms (biological process and molecular function categories) derived from down regulated genes at 3h, 12h, 16h and 24h.

3h (Treatment) vs 0h (control)		
Category	**Term**	**P-value**
GOTERM_MF_4	GO:0003700:transcription factor activity	0.004473946
GOTERM_BP_4	GO:0006350:transcription	0.004503343
GOTERM_BP_4	GO:0060255:regulation of macromolecule metabolic process	0.009129392
GOTERM_BP_4	GO:0080090:regulation of primary metabolic process	0.009859193
GOTERM_BP_4	GO:0019219:regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.009874439
GOTERM_BP_4	GO:0010556:regulation of macromolecule biosynthetic process	0.010286227
GOTERM_BP_4	GO:0051171:regulation of nitrogen compound metabolic process	0.010523796
GOTERM_BP_4	GO:0010468:regulation of gene expression	0.010984433
GOTERM_BP_4	GO:0031326:regulation of cellular biosynthetic process	0.013729337
GOTERM_BP_4	GO:0009889:regulation of biosynthetic process	0.014365233
12h (Treatment) vs 0h (control)		
Category	**Term**	**P-value**
GOTERM_BP_4	GO:0006259:DNA metabolic process	1.50E-05
GOTERM_MF_4	GO:0043566:structure-specific DNA binding	3.59E-04
GOTERM_BP_4	GO:0006350:transcription	0.004572353
GOTERM_BP_4	GO:0034645:cellular macromolecule biosynthetic process	0.021026247
GOTERM_BP_4	GO:0019219:regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.021180641
GOTERM_BP_4	GO:0010556:regulation of macromolecule biosynthetic process	0.022448487
GOTERM_BP_4	GO:0009404:toxin metabolic process	0.022569037
GOTERM_BP_4	GO:0051171:regulation of nitrogen compound metabolic process	0.02318764
GOTERM_BP_4	GO:0010468:regulation of gene expression	0.024636235
GOTERM_MF_4	GO:0030554:adenyl nucleotide binding	0.02952199
16h (Treatment) vs 0h (control)		
Category	**Term**	**P-value**
GOTERM_BP_4	GO:0006334:nucleosome assembly	0.002925583
GOTERM_BP_4	GO:0065004:protein-DNA complex assembly	0.003668595
GOTERM_BP_4	GO:0034728:nucleosome organization	0.003900115
GOTERM_BP_4	GO:0006325:chromatin organization	0.009020023
GOTERM_BP_4	GO:0007346:regulation of mitotic cell cycle	0.01500042
GOTERM_BP_4	GO:0009404:toxin metabolic process	0.016965373
GOTERM_MF_4	GO:0016818:hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	0.023087609
GOTERM_BP_4	GO:0051726:regulation of cell cycle	0.025982286
GOTERM_BP_4	GO:0048147:negative regulation of fibroblast proliferation	0.027009167
GOTERM_MF_4	GO:0030554:adenyl nucleotide binding	0.037126831
24h (Treatment) vs 0h (control)		
Category	**Term**	**P-value**
GOTERM_BP_4	GO:0000280:nuclear division	9.87E-08
GOTERM_BP_4	GO:0007067:mitosis	9.87E-08
GOTERM_BP_4	GO:0000087:M phase of mitotic cell cycle	1.15E-07
GOTERM_BP_4	GO:0022403:cell cycle phase	2.31E-06
GOTERM_BP_4	GO:0000279:M phase	2.89E-06
GOTERM_BP_4	GO:0048534:hemopoietic or lymphoid organ development	0.002704899
GOTERM_BP_4	GO:0002520:immune system development	0.003495867
GOTERM_MF_4	GO:0030554:adenyl nucleotide binding	0.007347879
GOTERM_BP_4	GO:0045596:negative regulation of cell differentiation	0.008367962
GOTERM_BP_4	GO:0030097:hemopoiesis	0.011324999

A visual summary of GO identifiers associated with up and down-regulated genes for each time point was created using REVIGO [19] and is presented in figure 3. This dataset lists gene functional categories expressed as a function of Epidermal growth factor (EGF) during the time course experiment and measured using the Affymetrix microarray platform. Figure 3 shows GO terms associated with up-regulated genes involved in regulation of programmed cell death, organ development, chemotaxis, epithelial cell differentiation, regulation of endocrine process, ectoderm development and positive regulation of mitosis. In contrast, GO terms associated with down-regulated genes were associated with DNA metabolism, immune system development, toxin metabolism, regulation of mitotic cell cycle, nucleosome assembly, regulation of cell proliferation and mitosis.

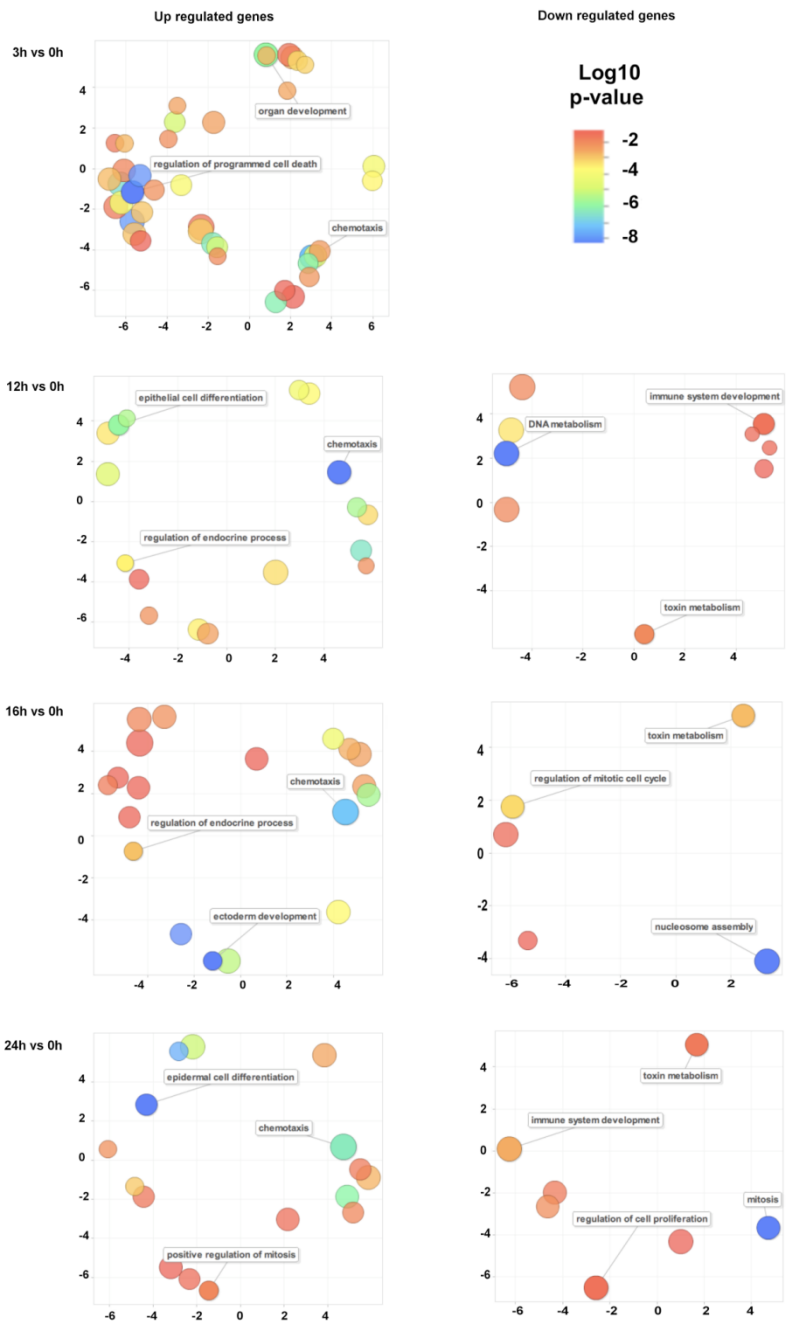

Figure 3: Statistically significant GO terms (Biological process and Molecular function categories) derived from up and down regulated genes at 3h, 12h, 16h and 24h. A visual summary of GO terms associated with down-regulated genes shows that there are no GO identifiers associated with down-regulated genes at 3h. Log10 p-value legend is in the upper right hand corner. Blue and green bubbles are GO terms with more significant **p**-values than the orange and red bubbles. Bubble size indicates the frequency of the GO term in the underlying GO database (bubbles of more general terms are larger). The bubble **x** and **y** coordinates were derived by applying multidimensional scaling to a matrix of the GO

terms' semantic similarities; consequently, their closeness on the plot should closely reflect their relationship in the GO graph structure

GO terms associated with differentially expressed proteins

GO enrichment analysis was performed using functional categories for the 50 proteins that were chosen based on the highest combined ion score in each spot to determine which biological process and molecular function were significantly over or under represented in a group of proteins in EGF treated A431 cells. The GO term analysis for these proteins was based on Fisher's exact test using the EASE modification ($p<0.05$) and multiple testing corrections were performed using the Benjamini-Hochberg FDR method. For GO terms of up and down-regulated proteins, we present Biological process and Molecular function categories, GO terms and their p-values for the 50 proteins with the highest ion score (Table 4).

Table 4: Statistically significant GO terms (biological process and molecular function categories) derived from 50 highest ion score proteins.

Category	Term	P-value
GOTERM_BP_4	GO:0019047:provirus integration	2.13E-04
GOTERM_BP_4	GO:0030069:lysogeny	2.13E-04
GOTERM_BP_4	GO:0046365:monosaccharide catabolic process	0.00102206
GOTERM_BP_4	GO:0019059:initiation of viral infection	0.001711347
GOTERM_BP_4	GO:0044275:cellular carbohydrate catabolic process	0.001719204
GOTERM_BP_4	GO:0019318:hexose metabolic process	0.001982684
GOTERM_BP_4	GO:0005996:monosaccharide metabolic process	0.003352058
GOTERM_BP_4	GO:0016052:carbohydrate catabolic process	0.003489178
GOTERM_BP_4	GO:0044262:cellular carbohydrate metabolic process	0.004285297
GOTERM_BP_4	GO:0019058:viral infectious cycle	0.00644651
GOTERM_MF_4	GO:0004866:endopeptidase inhibitor activity	0.006988913
GOTERM_MF_4	GO:0051287:NAD or NADH binding	0.007169294
GOTERM_BP_4	GO:0006096:glycolysis	0.007663101
GOTERM_BP_4	GO:0007163 : establishment or maintenance of cell polarity	0.008307263
GOTERM_BP_4	GO:0030836: positive regulation of actin filament depolymerization	0.008402681
GOTERM_BP_4	GO:0000902: cell morphogenesis	0.017153155
GOTERM_BP_4	GO:0007015: actin filament organization	0.017341027
GOTERM_BP_4	GO:0043436 :oxoacid metabolic process	0.018776192
GOTERM_BP_4	GO:0043243 : positive regulation of protein complex disassembly	0.019499464
GOTERM_BP_4	GO:0010638 : positive regulation of organelle organization	0.022647164
GOTERM_BP_4	GO:0032989 : cellular component morphogenesis	0.024499478
GOTERM_BP_4	GO:0030036 : actin cytoskeleton organization	0.025222517
GOTERM_MF_4	GO:0016616 : oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	0.032275158
GOTERM_BP_4	GO:0048513:organ development	0.047261031

Visualization of GO terms, associated with the 50 highest scoring proteins, revealed Proteins GO terms involved in provirus integration, establishment or maintenance of cell polarity, monosaccharide catabolism and positive regulation of actin filament depolymerization (Figure 4). This supported our finding that cytoskeletal destabilization is probably one of the causes of apoptosis resulting from EGF treatment [28].

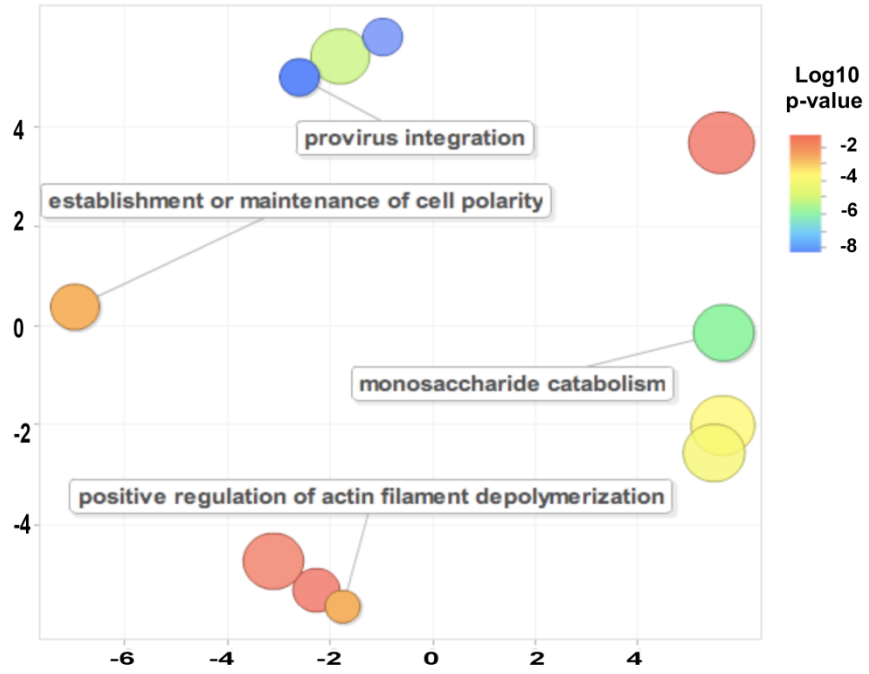

Figure 4: Statistically significant GO terms (Biological process and Molecular function categories) derived from 50 proteins with the highest ion score. Log10 p-value legend is in the upper right hand corner. Blue and green bubbles are GO terms with more significant **p**-values than the orange and red bubbles. Bubble size indicates the frequency of the GO term in the underlying GOA database (bubbles of more general terms are larger). The bubble **x** and **y** coordinates were derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities; consequently, their closeness on the plot should closely reflect their closeness in the GO graph structure.

Common domains of differentially expressed genes and proteins during EGF treatment

We extracted PFAM domains (Pfam accession or identifier) from 217, 251, 213, and 207 differentially expressed genes that were selected at 3 hours, 12 hours, 16 hours, and 24 hours

post EGF treatment respectively to determine if a set of genes/protein was related to a specific protein family. Table 5 and figure 5 show the number of Pfam accessions at each time point for up and down regulated genes, both annotated and un-annotated.

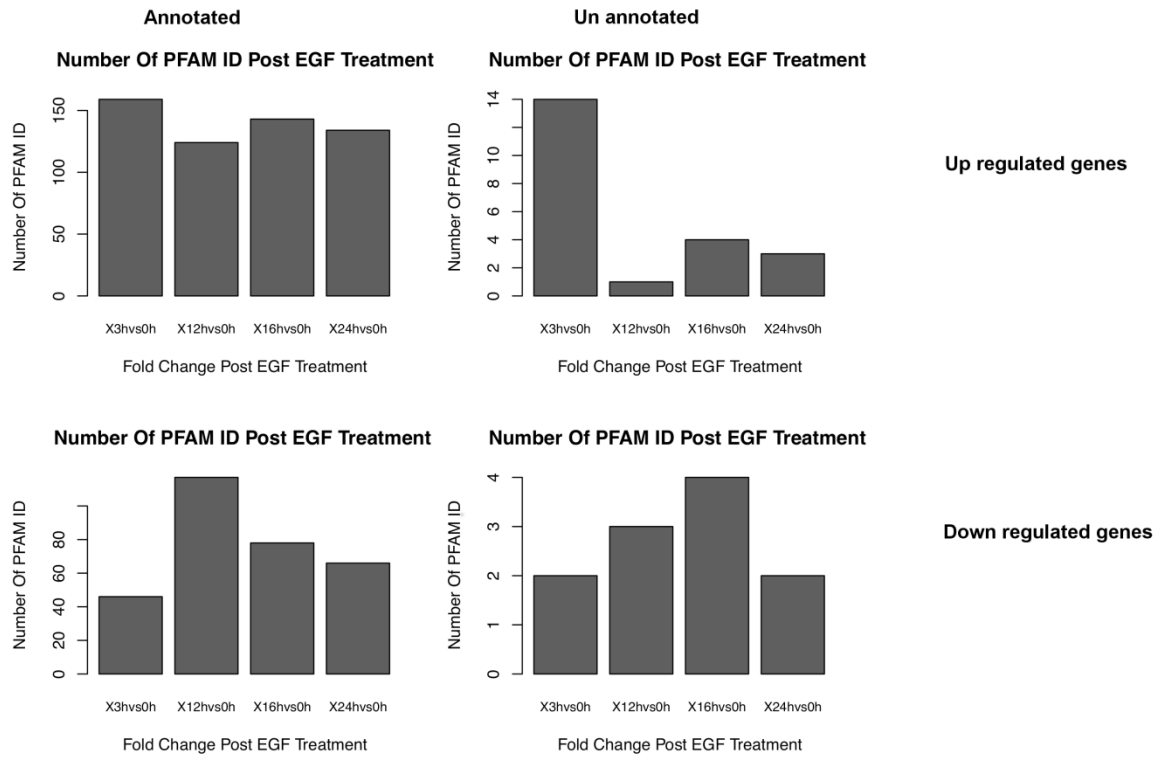

Figure 5: Number of PFMA ID for up and down regulated genes, annotated and un-annotated at each time point after EGF treatment.

Each Pfam accession was found in at least one time point supplementary Table S6. We extracted 69 PFAM domains (Pfam accession or identifier) from 50 proteins that were chosen based on their highest combined ion score in each spot (Table 5).

Domains shared between gene expression data and proteomics data

13 Pfam domains were shared between gene expression and proteomics data. Serpin (serine protease inhibitor) and S-100/ICaBP type calcium binding domains were over represented among these domains (Table 5).

Table 4: Number of Pfam accessions in annotated and un annotated genes in microarray and proteomics data along with the shared Pfam accessions.

Microarray gene expression

Pfam number in up regulated genes			**Pfam number in down regulated genes**	
Time	**Annotated**	**Un annotated**	**Annotated**	**Un annotated**
3h-0h	159	14	46	2
12h-0h	124	1	117	3
16h-0h	143	4	78	4
24h-0h	134	3	66	2

Proteomics protein level

50 significant proteins with highest ion score

Pfam number 69

	Proteomics protein level	**Microarray gene expression**				
		Intersect Pfam number				
pfam accession	**All time**	**3hvs0h**	**12hvs0h**	**16hvs0h**	**24hvs0h**	**Domain annotation**
PF00079	3	5	5	3	3	Serpin (serine protease inhibitor)
PF01023	1	1	3	4	5	S-100/ICaBP type calcium binding domain
PF00350	1	1	0	0	0	Dynamin
PF00373	1	1	0	0	0	FERM central domain
PF00412	1	1	1	1	1	LIM domain
PF00561	1	0	1	1	2	alpha/beta hydrolase fold
PF00578	1	1	1	1	1	AhpC/TSA family
PF00591	1	0	0	1	1	Glycosyl transferase family, a/b domain
PF02885	1	0	0	1	1	Glycosyl transferase family, helical bundle domain
PF03096	1	0	1	1	1	Ndr family
PF07831	1	0	0	1	1	Pyrimidine nucleoside phosphorylase C-terminal domain
PF09379	1	1	0	0	0	FERM domain
PF00036	1	1	0	0	1	EF hand

Functional classification

PANTHER classification software was used to assign identified genes and proteins to pathway and protein classes after EGF treatment (Table 6). Of particular interest were pathways shared between gene expression and proteomics data, including the EGF receptor signaling pathway, the Interferon-gamma signaling pathway and Inflammation mediated by chemokine and cytokine

signaling pathway. Others, such as Apoptosis signaling, TGF-beta signaling and Wnt signaling pathways were activated constitutively in gene expression data after EGF treatment. At 3h post EGF treatment in gene expression data, signalling pathways including FAS signaling pathway and JAK/STAT signaling pathway were observed. This suggests that these pathways were activated early during EGF treatment. Other signaling pathways such as the FGF signaling pathway, Pyruvate metabolism and Pentose phosphate pathways were found only in the proteomics data.

Classified proteins included kinases, receptors, transcription factors (T.Fs) and calcium binding proteins. Apoptotic kinases including MAP3K8, TRIB1, PLK3 and LYN were over expressed at 3h after EGF treatment. In the proteomics data, Pyruvate Kinase, ,muscle **(**PKM2) expression level increased dramatically during the EGF treatment. At the receptor level, we noticed that G protein-coupled receptor 132 (GPR132) was expressed constitutively, while tumor necrosis factor receptor superfamily, member 9 (TNFRSF9) and Hydroxycarboxylic Acid Receptor 2 (GPR109A) were expressed early during EGF treatment. At the late stage of EGF treatment, OSMR and IL13RA2 were expressed. No receptors were detected in the proteomics data. With respect to T.Fs, it is known that STATs and IRFs are involved in the induction of apoptosis in A431 cells [29]. Our data showed that STAT gene expression changed less than 2 fold while IRFs such as IRF9 were significantly over expressed during the time of treatment. The T.F. with the highest gene expression was PRDM1, involved in the regulation of apoptosis [30]. In the proteomics data, two T.Fs including CRIP2 and PRRK7 were observed. For calcium binding proteins, S100A8 and S100A9 were over expressed in gene expression data and S100A11 was over expressed in the proteomics data.

Table 6: PANTHER classification software was used to assign identified genes and proteins to pathway and protein classes. Proteins performe the functions in the cell and are therfore more relevant to the issue of cell life/death.

A- Pathways involved in induction of apoptosis post EGF treatment

	Microarray gene expression				**Proteomics**
Pathway ID	**3h-0h**	**12h-0h**	**16h-0h**	**24h-0h**	**All Time**
2-arachidonoylglycerol biosynthesis	Yes	No	Yes	No	No
Angiogenesis	Yes	No	No	No	No
Apoptosis signaling pathway	Yes	Yes	Yes	Yes	No
ANoon guidance mediated by semaphorins	Yes	No	No	No	No

ANoon guidance mediated by Slit/Robo	Yes	Yes	Yes	No	No
B cell activation	Yes	No	No	No	No
Blood coagulation	Yes	Yes	Yes	Yes	No
Cadherin signaling pathway	Yes	Yes	No	Yes	Yes
Cytoskeletal regulation by Rho GTPase	Yes	Yes	Yes	Yes	Yes
EGF receptor signaling pathway	Yes	Yes	Yes	Yes	Yes
Endothelin signaling pathway	Yes	No	No	No	No
FAS signaling pathway	Yes	No	No	No	No
Gonadotropin releasing hormone receptor pathway	Yes	Yes	Yes	Yes	Yes
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	Yes	No	No	No	No
Huntington disease	Yes	Yes	Yes	Yes	Yes
Inflammation mediated by chemokine and cytokine signaling pathway	Yes	Yes	Yes	Yes	Yes
Integrin signalling pathway	Yes	Yes	Yes	Yes	Yes
Interferon-gamma signaling pathway	Yes	Yes	Yes	Yes	Yes
Interleukin signaling pathway	Yes	Yes	Yes	Yes	No
JAK/STAT signaling pathway	Yes	No	No	No	No
ONoidative stress response	Yes	Yes	Yes	Yes	No
p53 pathway feedback loops 2	Yes	Yes	Yes	Yes	No
Parkinson disease	Yes	Yes	No	Yes	No
PDGF signaling pathway	Yes	No	No	No	No
Plasminogen activating cascade	Yes	Yes	Yes	Yes	No
T cell activation	Yes	No	No	Yes	No
TGF-beta signaling pathway	Yes	Yes	Yes	Yes	No
Threonine biosynthesis	Yes	No	No	No	No
Toll receptor signaling pathway	Yes	No	No	No	No
Wnt signaling pathway	Yes	Yes	Yes	Yes	No
5-HydroNoytryptamine degredation	No	Yes	Yes	No	No
5HT2 type receptor mediated signaling pathway	No	Yes	Yes	No	No
Alpha adrenergic receptor signaling pathway	No	Yes	Yes	No	No
Alzheimer disease-presenilin pathway	No	Yes	Yes	Yes	Yes
ANoon guidance mediated by netrin	No	Yes	No	No	No
Circadian clock system	No	Yes	No	Yes	No
DNA replication	No	Yes	No	No	No
Hedgehog signaling pathway	No	Yes	No	Yes	No
Histamine H1 receptor mediated signaling pathway	No	Yes	Yes	No	No
Ionotropic glutamate receptor pathway	No	Yes	Yes	Yes	No
Metabotropic glutamate receptor group III pathway	No	Yes	Yes	Yes	No
Nicotinic acetylcholine receptor signaling pathway	No	Yes	Yes	No	Yes
ONoytocin receptor mediated signaling pathway	No	Yes	Yes	No	No
p53 pathway	No	Yes	Yes	Yes	Yes
p53 pathway by glucose deprivation	Yes	Yes	Yes	Yes	No
P53 pathway feedback loops 1	No	Yes	Yes	Yes	No
Phenylethylamine degradation	No	Yes	Yes	No	No
Thyrotropin-releasing hormone receptor signaling pathway	No	Yes	Yes	No	No
Vasopressin synthesis	No	Yes	Yes	Yes	No
Pyrimidine Metabolism	No	No	Yes	Yes	Yes

Salvage pyrimidine deoNoyribonucleotides	No	No	Yes	Yes	Yes
Adenine and hypoNoanthine salvage pathway	No	No	No	Yes	No
Androgen/estrogene/progesterone biosynthesis	No	No	No	Yes	No
Purine metabolism	No	No	No	Yes	Yes
FGF signaling pathway	No	No	No	No	Yes
Glycolysis	No	No	No	No	Yes
Methylmalonyl pathway	No	No	No	No	Yes
Pentose phosphate pathway	No	No	No	No	Yes
Pyruvate metabolism	No	No	No	No	Yes
Succinate to proprionate conversion	No	No	No	No	Yes

B- Protein classes including kinases, receptors, transcription factors (T.Fs) and calcium binding proteins involved in the induction of apoptosis post EGF treatment.

	Microarray gene expression				**Proteomics**
Kinase ID	**3h-0h**	**12h-0h**	**16h-0h**	**24h-0h**	**All Time**
MAP3K8	Yes	No	No	No	No
PLK2	Yes	Yes	Yes	Yes	No
NUAK2	Yes	No	No	No	No
WEE1	Yes	No	No	Yes	No
TRIB1	Yes	Yes	Yes	No	No
PLK3	Yes	No	No	No	No
LYN	Yes	No	No	No	No
TRIB2	No	Yes	No	No	No
CSNK1G1	No	Yes	No	Yes	No
CIT	No	No	No	Yes	No
SAV1	No	No	No	Yes	No
CDKN3	No	No	No	Yes	No
PKM2	No	No	No	No	Yes
PGK1	No	No	No	No	Yes
	Microarray gene expression				**Proteomics**
Receptor ID	**3h-0h**	**12h-0h**	**16h-0h**	**24h-0h**	**All Time**
PLAUR	Yes	Yes	Yes	Yes	No
TNFRSF9	Yes	Yes	No	No	No
IL4R	Yes	No	No	No	No
ICAM1	Yes	Yes	No	No	No
ITGB8	Yes	No	No	No	No
IL1R2	Yes	Yes	Yes	Yes	No
IL1RL1	Yes	Yes	Yes	No	No
PTPN12	Yes	No	Yes	Yes	No
OR11H1	Yes	Yes	Yes	Yes	No
PTPRE	Yes	No	No	No	No
PVRL4	Yes	Yes	Yes	Yes	No
EDNRA	Yes	No	No	No	No
F2RL1	Yes	No	No	No	No
PTAFR	Yes	Yes	Yes	Yes	No

GPR109A	Yes	No	No	No	No
GPR132	Yes	Yes	Yes	Yes	No
LYN	Yes	No	No	No	No
NTN4	No	Yes	Yes	No	No
CCDC80	No	Yes	Yes	Yes	No
MXRA5	No	Yes	Yes	Yes	No
IL13RA2	No	Yes	Yes	Yes	No
SORT1	No	Yes	Yes	No	No
OSMR	No	Yes	Yes	Yes	No
GPRC5B	No	Yes	Yes	Yes	No
SCARA3	No	Yes	Yes	Yes	No
DLK2	No	Yes	No	No	No
BCAM	No	Yes	Yes	Yes	No
HEPHL1	No	No	Yes	Yes	No
PIK3IP1	No	No	No	Yes	No
EFEMP1	No	No	No	Yes	No

	Microarray gene expression				**Proteomics**
T.F ID	**3h-0h**	**12h-0h**	**16h-0h**	**24h-0h**	**All Time**
PRDM1	Yes	Yes	Yes	Yes	No
PHF17	Yes	No	No	No	No
RELB	Yes	No	No	No	No
HOXA5	Yes	No	No	No	No
BCL6	Yes	Yes	Yes	Yes	No
RNF19B	Yes	No	No	No	No
IRF6	Yes	No	No	No	No
BHLHE41	Yes	No	No	No	No
IRF1	Yes	No	No	No	No
JUB	Yes	No	No	No	No
GRHL1	Yes	Yes	Yes	Yes	No
ETV6	Yes	No	No	No	No
NLRC5	Yes	No	No	No	No
GRHL3	Yes	Yes	Yes	Yes	No
AIM2	Yes	Yes	Yes	Yes	No
CITED2	Yes	No	No	No	No
ZNF488	Yes	Yes	Yes	Yes	No
KBTBD6	Yes	No	No	No	No
KIAA1586	Yes	No	No	No	No
JUNB	Yes	No	No	No	No
OVOL1	Yes	Yes	No	No	No
SPRY4	Yes	No	No	No	No
TRIML2	Yes	No	No	Yes	No
ZNF267	Yes	No	No	No	No

MITF	Yes	No	No	No	No
OCLN	Yes	No	No	No	No
MAFK	Yes	No	No	No	No
IRF9	Yes	Yes	Yes	Yes	No
ZNF323	Yes	Yes	Yes	Yes	No
KLHL13	No	Yes	No	No	No
PER3	No	Yes	Yes	No	No
MXD1	No	Yes	Yes	Yes	No
CBX5	No	Yes	Yes	Yes	No
MYB	No	Yes	Yes	No	No
ID1	No	Yes	Yes	No	No
PER2	No	Yes	No	No	No
TP53	No	Yes	Yes	Yes	No
NFIB	No	Yes	No	No	No
DLK2	No	Yes	No	No	No
CITED4	No	Yes	No	Yes	No
MAML2	No	Yes	No	Yes	No
PBX1	No	Yes	Yes	Yes	No
MYBL1	No	Yes	Yes	Yes	No
ZNF114	No	No	Yes	Yes	No
PAX9	No	No	Yes	No	No
ZNF362	No	No	No	Yes	No
ZNF594	No	No	No	Yes	No
CRIP2	No	No	No	Yes	Yes
ZNF814	No	No	No	Yes	No
PARK7	No	No	No	No	Yes
	Microarray gene expression				**Proteomics**
Calcium binding proteins	**3h-0h**	**12h-0h**	**16h-0h**	**24h-0h**	**All Time**
MACF1	Yes	No	No	No	No
S100A3	Yes	No	No	No	No
FLNB	No	Yes	Yes	Yes	No
PLCE1	No	Yes	Yes	No	No
S100A8	No	Yes	Yes	Yes	No
S100A9	No	Yes	Yes	Yes	No
DLK2	No	Yes	No	No	No
S100A2	No	Yes	Yes	Yes	No
MICALCL	No	No	Yes	Yes	No
AGPAT9	No	No	Yes	No	No
S100A7	No	No	Yes	Yes	No

C1S	No	No	Yes	Yes	No
PIK3IP1	No	No	No	Yes	No
EFEMP1	No	No	No	Yes	No
GAS2L3	No	No	No	Yes	No
S100A12	No	No	No	Yes	No
CALML5	No	No	No	Yes	No
KLK9	No	No	No	Yes	No
EHD4	No	No	No	No	Yes
ANXA2	No	No	No	No	Yes
S100A11	No	No	No	No	Yes

Discussion

It is known that a high concentration of EGF is capable of inducing apoptosis in A431 cells that express vast numbers of HER1 receptor [5-11]. Understanding the induction of apoptosis via the activation of HER1 receptor may lead to discovery of new drugs to regulate apoptosis in HER1-expressing cancers. In order to further understand the mechanism of action EGF induced apoptosis global analyses of gene expression and associated regulatory miRNAs and protein level using microarray and proteomics platforms were carried out. This chapter focuses on the analysis of the gene expression and protein expression data with respect to annotations and known functions. Our results showed that gene expression and proteomics data were quite different, with very little overlap in terms of transcripts and proteins. Initial gene expression (at 3hrs post EGF) was significantly different to later gene expression time points, which had much more similar profiles, as observed in Figure. 1. This means that the biggest changes in gene expression occurred prior to the early stage of induction of apoptosis. This was in contrast to the proteomics data, which revealed that the earlier time points (up to and including 24h post EGF) had more similar expression profiles than the late time point (Figure 2).

It was previously shown that EGF could activate the interferon/JAK/STAT signaling pathway in A431 cells. This pathway not only responds to EGF but also to interferon (IFN-gamma), and is known to cause cell cycle arrest and death through the caspase pathway [31]. Our published data showed the involvement of additional downstream components of the interferon/JAK/STAT signaling pathway that may be part of the induction of apoptosis in A431 cells [28]. Furthermore, our PANTHER pathway analysis revealed that the JAK/STAT signaling pathway was activated

only at the early stage of EGF treatment in the gene expression data while interferon–gamma signaling was observed in both gene expression and proteomics data (Table 6).

The IFN-gamma pathway also plays a role in the activation of the Serpin family, specifically in the production of SerpinA1. SerpinA1 production by invasive cutaneous squamous cell carcinoma (SCC) cells was dependent on p38 mitogen-activated protein kinase activity and was up-regulated by EGF and IFN-gamma [32]. Our published data [28] and domain analysis showed that SERPINB3 and SERPINB1 are the only members of this family present in both the gene expression and proteomics data. SERPINB3 has been shown to have an anti-apoptotic function [33] whereas SERPINB1 can be either anti-apoptotic or pro-apoptotic once it has been cleaved to produce L-DnaseII [34]. L-DnaseII, is capable of DNA degradation and can induce apoptosis in a caspase-independent fashion [35, 36]. We were able to show that EGF induced SERPINB1 was cleaved to produce the pro-apoptotic L-DnaseII product [28].

In addition to induction of caspases via JAK/STAT and caspase independent apoptosis by SERPINB1, apoptosis can be induced by cytoskeletal destabilization. Evidence suggests that depolymerization of the actin cytoskeleton or alteratation of actin filament dynamics is sufficient to induce programmed cell death in yeast and some animal cells, depending on the cell type [37]. GO term analysis of our proteomics data showed that several proteins with significant changes in expression were associated with positive regulation of actin filament depolymerisation (Figure 3, Table 4). Therefore, our published data supports a role for cytoskeletal destabilization as one of the ultimate causes of apoptosis resulting from EGF treatment [28].

Other factors can regulate cytoskeletal organization, specifically calcium-binding proteins are known be involved in the induction of apoptosis and regulation of actin organization and actin filament dynamics. In the context of apoptosis, we observed induction of gene expression for S100A8 and S100A9, which are capable of inhibiting growth and inducing cell death in many tumor cells such as MM46 mouse mammary carcinoma and MCF-7 human mammary adenocarcinomas [38]. We also observed increased protein levels for S100-A11, which is capable of activating, $P21^{WAF1/CIP1}$, a negative regulator of cell growth in human epidermal keratinocytes [14]. This is of interest because it has also been reported that decreased levels of

S100A11 protein are associated with various types of cancer, including breast [39] and prostate cancer [40]. Therefore, S100-A11 may be a superior candidate for inducing apoptosis.

In the context of actin organization and actin filament dynamics, it is known that the calcium-binding protein S100-A11 (S100C/A11) is capable of binding to actin through the COOH-terminal region [41] and might control actin organization and have a significant influence on cell morphology. It is therefore possible that cytoplasmic S100-A11 is implicated in the regulation of actin organization and actin filament dynamics [42]. This is consistent with our finding that S100-A11 protein expression increased dramatically post EGF treatment along with differentially expressed cytoskeletal components and cell adhesion proteins as observed in both transcript and protein levels. This indicates a likely role for EGF induced cytoskeletal destabilization as a trigger for apoptosis. This was reinforced by our domain analysis, that showed the Ca^{++} binding domain PF01023, present in at least 21 different types of S100 proteins [43], was the second most common PFAM identifier (Table 4) found in both EGF induced transcripts and proteins.

In addition , Apoptotic kinase, LYN was over expressed at 3h after EGF treatment.
there is a connection between Lyn kinase and EGFR/HER1. For instance, the importance of Lyn to EGFR/HER1 signaling and cell viability was investigated by treatment of Calu3 with Lyn specific silencing RNAs. As a result, declined Lyn expression and phosphorylation readily inhibited Y-1068 autophosphorylation of EGFR [44].

Because our gene expression and proteomics data showed little overlap, yet there were common pathways found in both, we felt that post-transcriptional regulation might be important. MicroRNAs are known to be important post-transcriptional regulators; therefore, we sought to identify potential regulatory miRNAs with respect to induction of cell death by EGF. Our submitted paper (chapter 3) showed that eight miRNAs known to target the interferon pathway were found to be down regulated, consistent with a novel layer of regulation of interferon pathway components downstream of JAK/STAT.

We have shown that using multiple, complimentary platforms such as gene expression, proteomics and miRNA expression to analyse the induction of apoptosis in A431 cells as a result of EGF treatment, we may be able to better understand the molecular mechanisms at work. This in turn may suggest strategies for combined therapies and/or predict novel potential mechanisms/targets for chemotherapy aimed at inducing cell death in cancer cells.

Methodology

Cancer Cell Line

A431 was (epidermoid vulva carcinoma) cell line was purchased from the ATTC (Manassas,VA) and utalized in this study. Cells were cultured at 37°C in a humidified 5% CO2 atmosphere. Dulbecco's modified Eagle's medium, 10% fetal calf serum, and 4 mM L-glutamine (Cambrex Bio Science).

Transcriptomics Profiling of Apoptosis Derived from High Concentration of EGF in A431 cells

Cell culture, mRNA isolation and microarray analysis performed as described in [28].

Proteomics Profiling of Apoptosis Derived from High Concentration of EGF in A431 cells

Cell culture, protein isolation and proteomic profiling carried out as described in [28].

Clustering of Microarray and Proteomics Data

We used the average complete linkage method for clustering of the microarray and proteomics expression data by R language (http://www.r-project.org/). This algorithm measures the average distance between the members of different clusters. HeatMap represented the clusters and the relationship between them at each time point.

Gene Ontology (GO) Analysis, Summarization and Visualization

To capture an overview of the functional behavior of A431 cell line during the course of apoptosis, the significant over expressed, down expressed genes and proteins were assigned to the functional categories using DAVID Bioinformatics Resources6.7 [18]. Significant over-represented GO groups were selected based on Fisher' s exact test using the EASE modification ($P < 0.05$) and multiple testing corrections was performed using Benjamini- Hochberg FDR method. Then, REVIGO was used to summarize and visualize a long list of GO terms in each time point in the cluster and to reduce functional redundancies to assist in interpretation. Its algorithm relies on semantic similarity measures.

Pfam Analysis

To capture an overview of the Pfam domains (PFAM accession number of identifier) in A431 cell line during the course of apoptosis, Biomart package from Bioconductor (http://www.bioconductor.org) were used extract PfAM ID from the significant over expressed, down expressed genes and proteins from microarray and proteomics experiments respectively.

Gene and Protein Classification According to Gene Ontology

The PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification software was used to assign identified genes and proteins according to pathway and Protein classes [45].

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CHAPTER FIVE

Conclusion and Future Directions

Breast cancer is one of the major causes of death in women around the world. Overexpression of epidermal growth factor receptor (EGFR) is correlated with loss of estrogen receptor and poor prognosis in breast cancer [1]. We used A431 cells that over expressed HER1 as a model to study breast cancer that over expressed HER1which have been found in 30-40% of breast carcinomas and correlated with poor prognosis [2-4]. In contrast, MCF7 cells are estrogen receptor-positive [1] and have low levels of HER1-expressed receptor [4] making them less suitable for these experiments.

Previous investigators have shown that using a high dose of EGF will cause apoptosis in this cell line via the activation of the EGFR/HER signalling pathway. However, little work has been done to identify the underlying molecular mechanisms. Therefore, this thesis was carried out to identify downstream components by performing global analyses of gene expression and associated regulatory miRNAs and protein levels using microarray and proteomics platforms and network prediction via the activation of HER receptors.

This thesis has investigated underlying molecular mechanisms of apoptosis induction by EGFR/HER1. Detailed understanding of EGFR/HER1 signaling-based apoptosis, in particular regulatory mechanisms and genetic network hierarchies that we have predicted can lead to discovery of new drug targets to regulate apoptosis in cancers that over-express HERs. In fact, the backbone of molecular activity/suppression can be monitored by molecular networks [5]. This thesis is divided into 5 chapters. Chapter 1 addressed the brief introduction that includes general scope about breast cancer and the contribution of genetic factors to it, research hypothesis and the gape tat require to be fulfilled using various assay and platforms; and showed literature review of breast cancer. The first paper, titled “Combined gene expression and proteomic analysis of EGF induced apoptosis in A431 cells suggests multiple pathways trigger apoptosis” is reproduced in chapter 2. This chapter shows that a high dose of EGF, leading to the induction of apoptosis in A431 cells can activate a number of pathways known to promote apoptosis. Because more than one pathway may be required to trigger apoptosis in these cells,

targeting a combination of these pathways could be an alternative approach to induce apoptosis in cancer cells. The second paper is titled "MicroRNAs are part of the regulatory network that controls EGF induced apoptosis, including elements of the JAK/STAT pathway, in A431 cells" and is reproduced in chapter 3. This paper indicates that miRNAs that target pro-apoptotic genes tend to decrease in abundance while their targets increase and that other miRNAs that target anti-apoptotic genes increase in abundance while their targets decrease. A number of miRNAs that would normally target interferon pathway induced pro-apoptotic transcripts were found to be down regulated, leading us to conclude that miRNAs may provide a novel layer of regulation within the interferon pathway, specifically with respect to components downstream of JAK/STAT. Finally, we speculate that a pull/push mechanism exists to favour the expression of pro-apoptotic genes as a result of high dose EGF treatment. Chapter 4 is titled "A Comprehensive Catalogue of Differentially Expressed Genes and Proteins From Epidermal growth factor (EGF) induced Apoptosis in A431 cells". This chapter presents a comprehensive catalogue of EGF regulated genes leading to apoptosis in A431 cell. This catalogue includes gene clustering analysis, gene ontology (GO) terms, protein domain and functional classification.

As it mentioned above, we used A431 cells that over expressed HER1 as a model to study breast cancer that over expressed HER1which have been found in 30-40% of breast carcinomas and correlated with poor prognosis [2-4]. The result can be translate to breast cancer cell line. For instance, A431 cells and MDA-MB-468 over expressed HER1 can be inhibited and undergo apoptosis by EGFR TK inhibitor ZD1839 [6]. I have not come cross any article mentioned that MDA-MB-468 may be undergo apoptosis via EGF stimulation . We can stimulate MDA-MB-468 cells that over expressed HER1 with EGF and examine the proliferation and apoptosis induction in these cells.

Several critical proteins including PDIA3, PKM and S100-A11 that are up regulated in A431 cells treated with EGF. Several articles indicate that over expression of PDIA3 (ERp57) leads to apoptosis via the activation of caspase-3 in endothelial cells [7] and prostate cancer [8]. It has been observed that PDIA3 is down regulated in tumor breast cancer cell lines compared to non-tumor forming lines [9]. Our results show that the protein level of PDIA3 is low in A431 cell line at time 0 (control). However, the expression of this protein is sharply increased after EGF treatment. As a result, this protein is an excellent candidate as a trigger for apoptosis.

It has been observed that M2-PK inhibition rescues cells from glucose starvation induced apoptotic cell death by increasing metabolic activity [5]. The protein level of this protein is down regulated in colon cancer cells [10]. It has been suggested that the elevation of intercellular levels of pyruvate may provide a potential therapeutic strategy in the treatment of cancer [10]. However, it has been observed that M2-PK expression is significantly higher in patients surviving breast cancer for more than 13 years [11]. Our results indicate that the protein level of M2-PK is almost doubled by EGF. This may be a good candidate for inducing apoptosis.

S100-A11 is capable of liberating SP1/SP3 from nucleolin and that free SP1/SP3 transcriptionally activates P21WAF1/CIP1, a negative regulator of cell growth in human epidermal keratinocytes [14]. Several articles mention that the protein level of S100-A11 is down regulated in various types of cancer including breast [12] and prostate cancer [13]. Our results show that this protein is up regulated dramatically after EGF induction. So, S100-A11 may also be a superior candidate for inducing apoptosis.

Our work has comprehensively identified downstream microRNAs/genes/proteins that are more likely to be useful targets for chemotherapy inducing cell death in cancer. The results from the comparative analyses and regulatory networks have provided valuable information and can be used as a starting point for functional validation of the multiple targets we have identified.

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Supplemental Materials

The attached CD-ROM/DVD-ROM contains supplemental documents and tables for chapter 2, chapter 3 and chapter 4.

For chapter 2, there are 3 supplemental tables.

For chapter 3, there are 2 supplemental tables.

For chapter 4, there are 6 supplemental tables.

Appendix 1: Additional materials for chapter two.

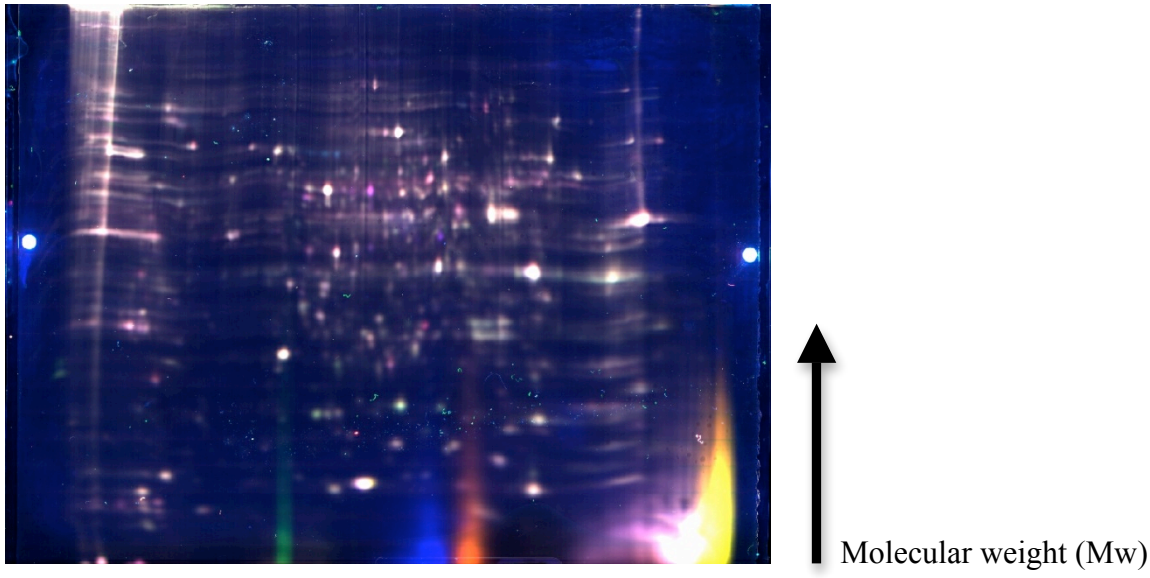
9 gels were utilized for time course analysis of A431 cells after EGF treatment.

Name	Cy3	Cy5	Cy2
Gel01	0-II	12-I	IPS
Gel02	3-II	3-I	IPS
Gel03	12-II	16-I	IPS
Gel04	16-II	24-I	IPS
Gel05	24-II	48-I	IPS
Gel06	48-II	0-I	IPS
Gel07	0-III	16-III	IPS
Gel08	3-III	24-III	IPS
Gel09	12-III	48-III	IPS

*IPS stands for internal protein standard.

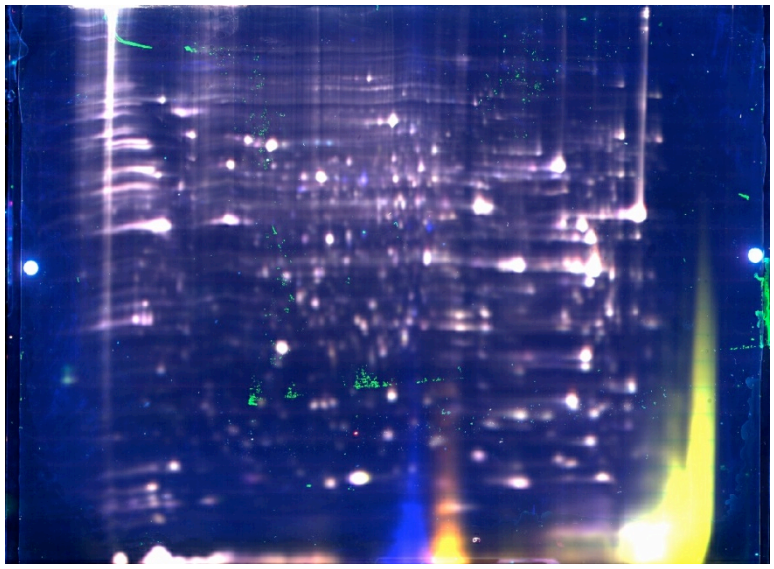
Gel 1

PH3 PH11



Gel 2

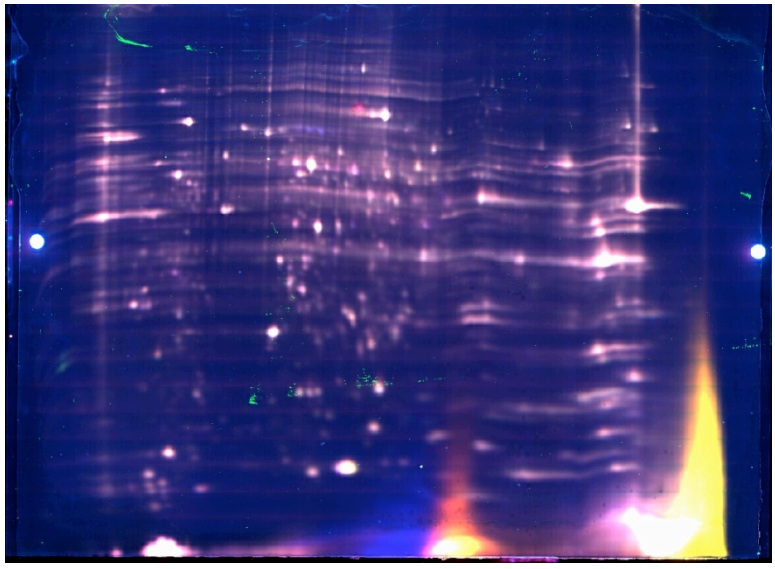
PH3 PH11



Molecular weight (Mw)

Gel 3

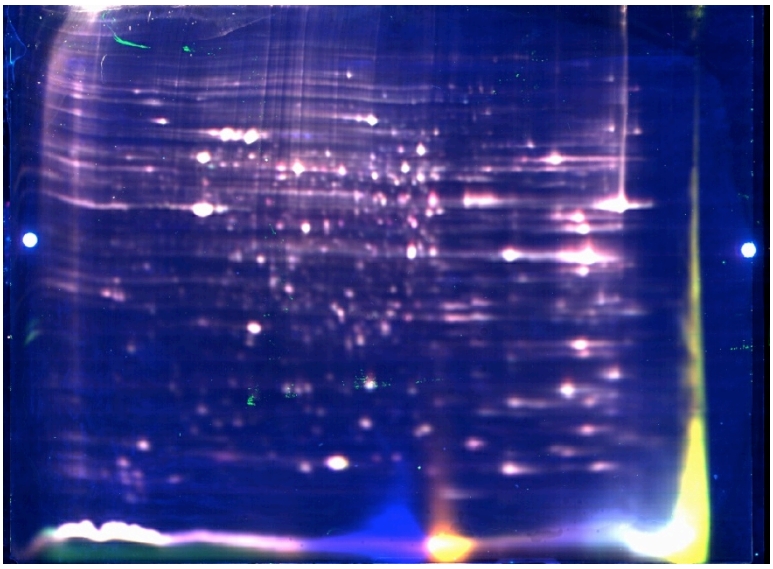
PH3 PH11



Molecular weight (Mw)

Gel 4

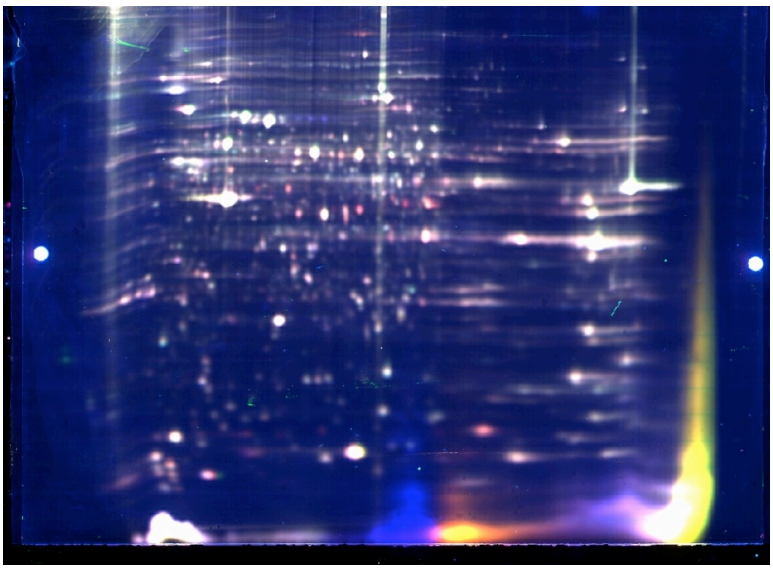
PH3 PH11



Molecular weight (Mw)

Gel 5

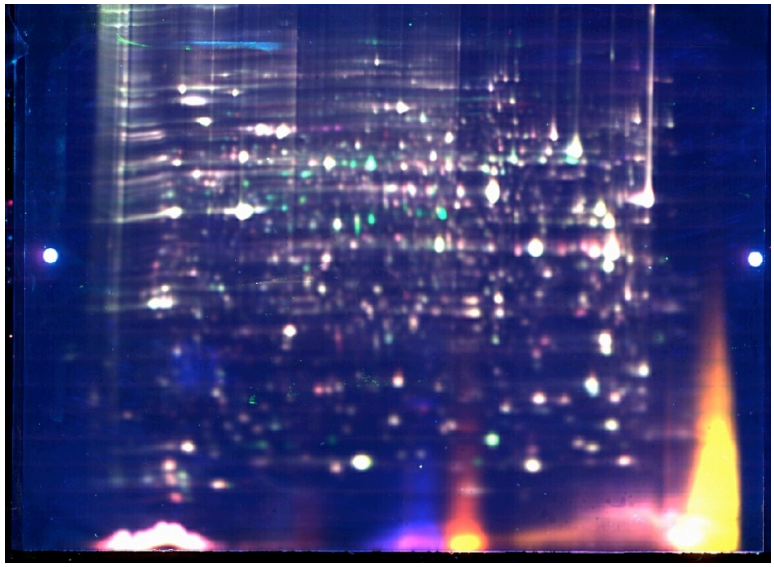
PH3 PH11



Molecular weight (Mw)

Gel 6

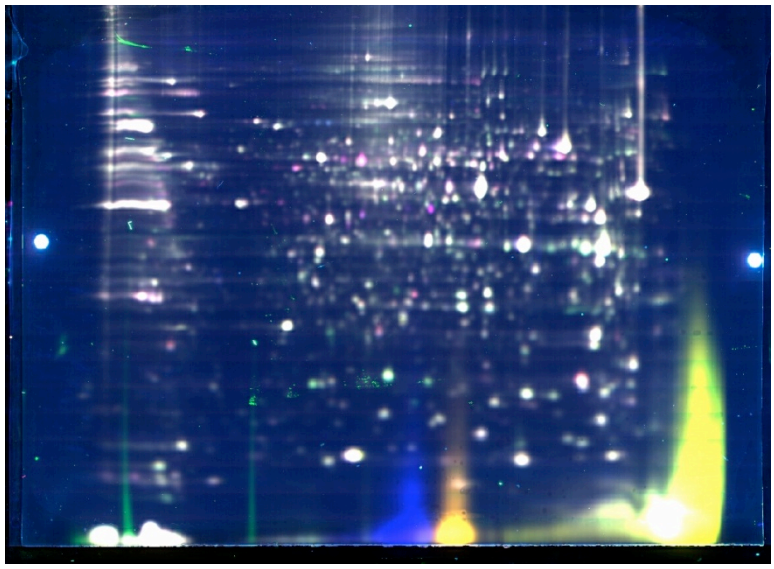
PH3 PH11



Molecular weight (Mw)

Gel 7

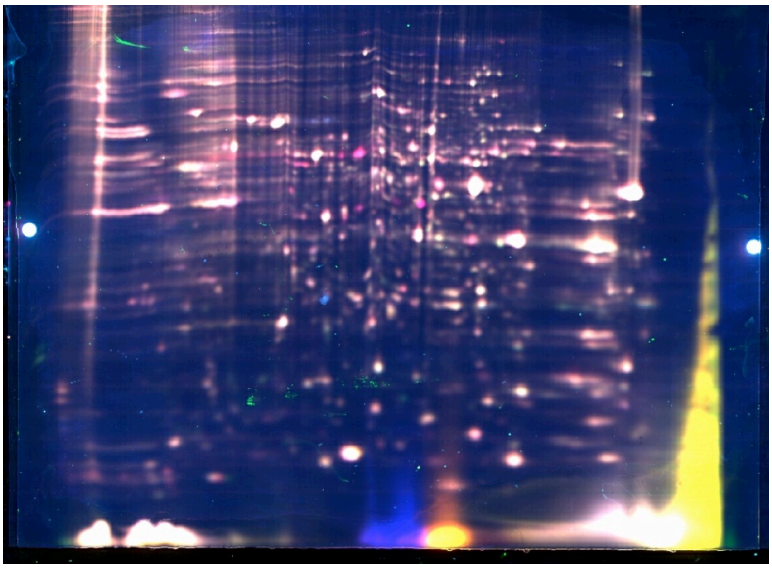
PH3 PH11



Molecular weight (Mw)

Gel 8

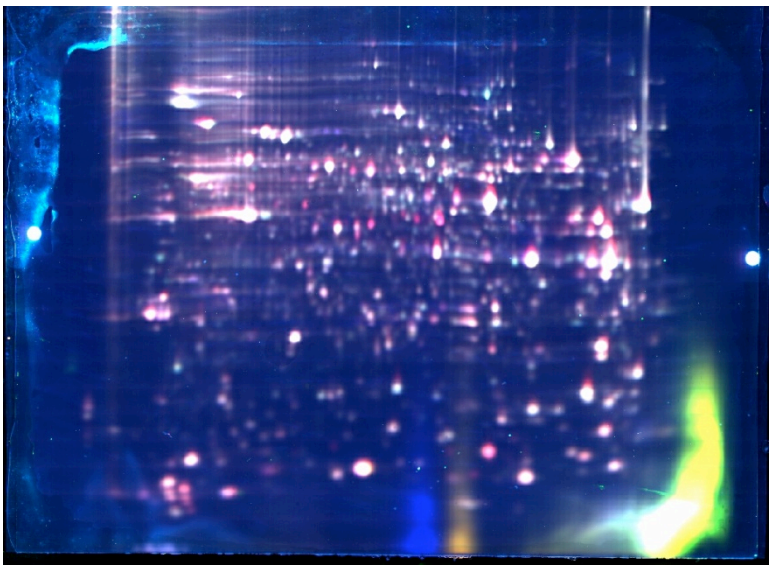
PH3 PH11



Molecular weight (Mw)

Gel 9

PH3 PH11

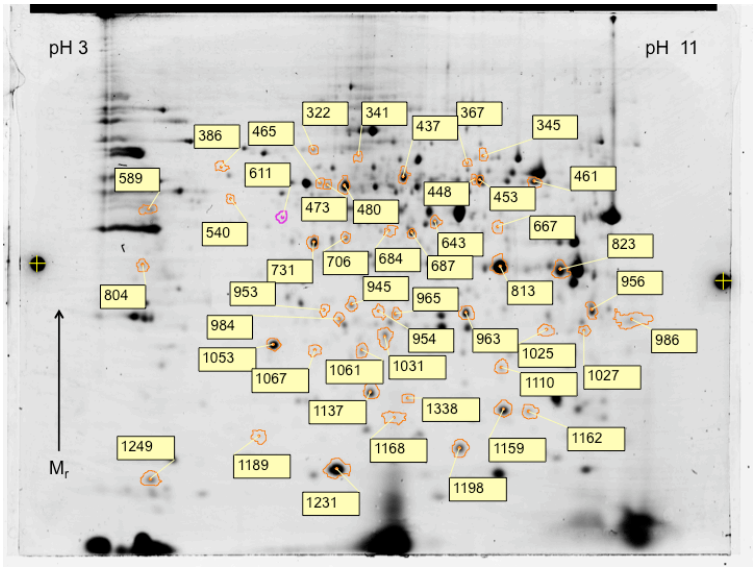


Molecular weight (Mw)

Picking spots from 9 gels that utilized for time course analysis of A431 cells after EGF treatment.

64 spots based on differential spot intensity values and expression patterns

A) 48 spots



B) 16 spots