Post-transcriptional regulation of Estrogen Receptor-α by miR-17-92 interaction and LMTK3 phosphorylation in Breast Cancer

BY

JIMMY JACOB

A thesis submitted for the degree of Doctor of Philosophy at Imperial College London

Department of Oncology, 1st floor ICTEM
Imperial College London
Hammersmith Hospital, Du Cane Road
London, W12 0NN

Supervisor: Prof. Justin Stebbing
Co-Supervisor: Dr. Leandro Castellano

Dedicated to my family

Abstract

Estrogen receptor-α (ERα) is expressed in two-thirds of BCs and is a well-known prognostic and predictive marker. For this reason it is one of the most studied proteins in BC. To understand how ERα positive BC develops, it is crucial to investigate both how this protein is regulated and which genes are modulated by it. MicroRNAs (miRNAs) control gene expression post-transcriptionally by interacting through sequence complementarity to their target transcripts. Through a microarray approach, we identified the subset of miRNAs modulated by ERα, that include up-regulation of miRNAs derived from the processing of two paralogous primary (pri-) transcripts, pri-miR-17-92 and pri-miR-106a-363. Characterisation of the miR-17-92 locus confirmed that the ERα target protein c-MYC binds its promoter in an estrogen-dependent manner. These findings indicated that miRNAs derived from these pri-miRNAs (miR-18a, miR-19b and miR-20b) target and down-regulate ERα, whilst a subset of pri-miRNA-derived mature miRNAs inhibit protein translation of the ERα transcriptional p160 co-activator, AIB1. Therefore, different subsets of the miRNAs identified act as part of a negative autoregulatory feedback loop. We observed that levels of pri-miR-17-92 increase earlier than the mature miRNAs derived from it, implicating precursor cleavage modulation after transcription. Pri-mir-17-92 is immediately cleaved by Drosha to pre-miR-18a, indicating that its regulation occurs during the formation of the mature molecule from the precursors.

Furthermore, we wanted to explore the new kinases that regulate the ERα activity. Thereby, we performed kinome screening (by RNAi technologies) to

determine kinases that regulate ERα in MCF-7 BC cells and identified a novel kinase, LMTK3, which acts as positive regulator of ERα's transcriptional activity. This could be a new therapeutic target and/or a novel biomarker for BC, although further studies are required to validate this. Together, these studies identify new transcriptional and translational factors that regulate ERα expression in BC.

Statement of originality and Declaration

All the experimentation presented and written in this thesis has been conducted by me, apart from the following:

Results section 3.1:
Microarray was done in collaboration with Genomics Laboratory, Hammersmith Hospital, Imperial College London. Data analysis was performed by Dr. Leandro Castellano. ChIP assay was performed together by Dr. Leandro Castellano and me. Luciferase activity assays were performed together by Dr. Leandro Castellano and me. JP13 cell lines for microarray and ERα constructs for luciferase activity were kindly provided by Dr. Laki Buluwela. Primers were designed by Dr. Leandro Castellano. Most of this work was published by us in [1].

Results section 3.2: Mass Spectrometry and analysis was done in collaboration with Dr. Robin Wait, Charing Cross Hospital, Imperial College London. EMSA and Northern Blotting were performed together by Dr. Leandro Castellano and me.

Results section 3.3: Kinome screening was was performed together by Dr. Georgios Giamas and me. Screening work was later published by us in [2]

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Publications

All the publications below have been published during my Ph.D and publications highlighted with * are included at back of the thesis.

Pinho FG, Frampton AE, Nunes J, Krell J, Alshaker H, **Jacob J**, Pellegrino L, Roca-Alonso L, de Giorgio A, Harding V, Waxman J, Stebbing J, Pchejetski D, Castellano L. Downregulation of microRNA-515-5p by the estrogen receptor modulates sphingosine kinase-1 and breast cancer cell proliferation. Cancer Res. 2013 Aug 8. [Epub ahead of print].

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Abbreviations

ADARs - Adenosine Deaminase Acting on RNA enzymes

AF1/AF2 - Activation function 1/Activation function 2

AIs - Aromatase Inhibitors

AIB1 - Amplified In Breast cancer 1

APS - Ammonium persulphate

ARS2 - Arsenic resistance protein 2

ATM - Ataxia-telangiectasia mutated

ATP - Adenosine triphosphate

BRCA1 – Breast cancer type 1 susceptibility protein

BRCA2 - Breast cancer type 2 susceptibility protein

BSA - Bovine Serum Albumin

CDK - Cyclin-dependent kinase

ceRNAs – Competing endogenous RNAs

c-MYC - C-myelocytomatosis

DBD - DNA-binding domain

DEAD box - Amino acid sequence D-E-A-D asp-glu-ala-asp

DGCR8 - DiGeorge syndrome critical region gene 8

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - Dimethyl Sulphoxide

DNA - Deoxyribonucleic acid

DNase - Deoyribonuclease

dsRBD - double-stranded RNA-binding domain

DSS - Double charcoal stripped serum

DTT – Dithiothreitol

E2 - 17β-Estradiol

ECL - Enhanced Chemi-Luminescence

EDTA - Ethylenediaminetetraacetic acid

EGFR – Epidermal growth factor receptor

ERα - Estrogen Receptor alpha

ERE - Estrogen response elements

ERK1/2 - Extracellular regulated kinases1 and 2

FCS - Foetal calf serum

GREB1 - Gene regulated in breast cancer 1

HCC - Hepatocellular carcinoma

HER2 - Human Epidermal growth factor Receptor 2

hnRNP A1 - heterogeneous nuclear ribonucleoprotein A1

hnRNP E2 - heterogeneous nuclear ribonucleoprotein E2

KH - hnRNP K homology domains

KSRP - KH-type splicing regulatory protein

LBD - Ligand binding domain

M9 - nucleocytoplasmic shuttling signal sequence

MAPK - Mitogen-activated protein kinase

MCF-7 - Michigan Cancer Foundation -7

miRNA – microRNA

mRNA – messenger RNA

PARP – Poly (ADP-ribose) polymerase Inhibitors

PBS - Phosphate Buffered Saline

PCR - Polymerase chain reaction

PKC - Protein kinase C

Pol II - RNA polymerase II

PR - Progesterone

PTEN - Phosphatase and tensin homologue

RanBP - ran-binding protein

RBPs - RNA binding proteins

RGG - Arg-Gly-Gly box

RNAi - RNA interference

RISC – RNA induced silencing complex

RRM - RNA Recognition Motifs

SDS - Sodium dodecyl sulphate

SERM - Selective ER modulator

siRNA - small interfering RNA

SRC1 - Steroid receptor coactivator 1

TBS - Tris buffered saline

TGFβ - Transforming Growth Factor beta

TIF2 - transcriptional mediators/intermediary factor 2

TRIM-NHL - Tripartite motif (consisting zinc fingers of both RING type and B Box type and a coiled-coil domain) NHL repeats.

TUT4 - Terminal Uridylyl Transferase 4

UCG - Ultraconserved gene

UTR – Untranslated region

Chapter 1: Introduction

1.1 Introduction

Cancer is caused by the uncontrolled proliferation and inappropriate survival of damaged cells. Healthy cells have developed safeguards to ensure that cell division, differentiation and death occur in a timely and coordinated fashion, both during foetal development and throughout life. Loss of control at cell cycle checkpoints can ultimately result in uncontrolled and abnormal growth of cells, often resulting in their migration to other tissues. When abnormal cells migrate to other tissues in the body, this process is known as metastasis [3, 4]. Most cancer-associated mortality is due to metastasis and yet it remains the most poorly understood element of cancer pathogenesis [5]. Loss of function of a tumour suppressor gene, or activation of an oncogene, is often responsible for the loss of cell cycle control and subsequent control of cellular proliferation leading to cancer progression [6-9].

1.1.1 Global Cancer Incidence

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries [10]. In 2008 there were more than 156,000 cancer-related deaths in the UK, accounting for 1 in 4 (27%) of deaths from all causes. Primary malignancies of the lung, bowel, breast and prostate account for almost half of all cancer related deaths in the UK [11]. (An aging population and the "modern lifestyle" of alcohol, cigarettes and processed foods are likely responsible for the increasing incidence and increase in the global burden of cancer [12].

It is estimated that 12.7 million cancer cases and 7.6 million cancer deaths have occurred worldwide in 2008, with 64% of deaths being in the economically developing world [13]. The incidence of all new cancer cases in economically developing countries is nearly double that of developed countries [12, 13]. Worse survival is likely to be related to a combination of a late stage at diagnosis and limited access to optimal treatment. Public education on diet and alcohol moderation, vaccinations (eg. human papilloma virus vaccines) and smoking cessation campaigns could all reduce the burden of cancer globally [12]. However, the treatment of aggressive and metastatic cancer requires the on-going endeavours of scientists and clinicians to fully understand the cancer cell biology and identify novel targets for treatment.

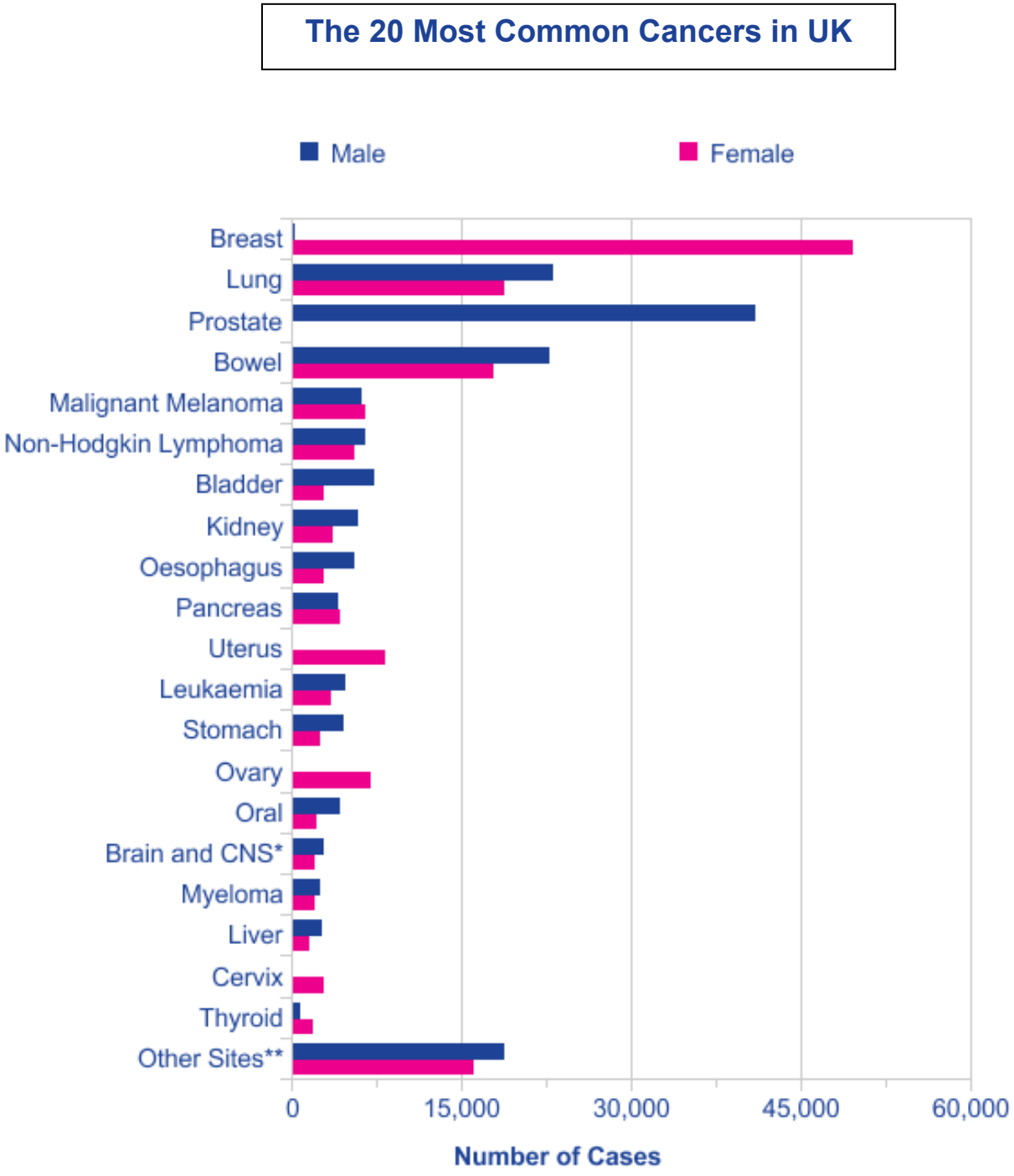


Figure 1: The most common diagnosed cancer cases in UK (excluding non-melanomatous skin cancers), 2010. The figure shows the number of males and females affected by different types of cancer per year in the UK. (www.cancerresearchuk.org)

1.1.2 Breast Cancer Incidence

Globally, breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of all new cancer cases and 14% of cancer deaths. In economically developing countries, it has now overtaken cervical cancer as the most common cause of cancer-related death in women [12-14]. In the UK it is also the most commonly diagnosed cancer in women (Fig. 1), and is the third most common cause of cancer-related death (after Bowel and Lung cancer) [15]. Approximately 45,000 new diagnoses of breast cancer are made in the UK every year and approximately 12,000 patients will die annually of their disease [16]. National screening programmes resulting in earlier detection, more streamlined clinical services and better therapies have resulted in a marked reduction in the annual death rate since the 1980s [17].

1.2 Breast Cancer

Risk factors for developing breast cancer include obesity, alcohol consumption, radiation, Lifelong Estrogenic Exposure and exposure to different carcinogens [17, 18], as well as genetic susceptibility. 10% of all breast cancers are thought to be hereditary [19], in which a the inheritance of a mutated gene predisposes the individual to the development of breast cancer. The most common genetic aberrations seen in breast cancer are in BRCA1 (breast cancer type 1 susceptibility protein) and BRCA2 (breast cancer type 2 susceptibility protein) [20-23]. BRCA1 is located on chromosome 17q21 and BRCA2 is located on chromosome 13q12.3. BRCA1 and BRCA2, which are both tumour suppressor

genes, are involved in multiple cellular functions like DNA damage repair, maintaining genomic integrity and transcriptional regulation. Mutations within BRCA1 and/or 2 leads to translation of truncated proteins that lack normal function and are unable to perform their usual function of homologous recombination and DNA repair properly, hence increasing the risk of accumulating other genetic mutations due to DNA damage and hence promoting breast cancer genesis [22, 24].

1.2.1 Biomarkers in Breast cancer

Biomarkers are often useful in clinical practice. A prognostic biomarker may suggest how a cancer is going to behave, whilst a predictive biomarker can inform clinicians about the likelihood of a cancer responding to a particular treatment. The Estrogen Receptor (ER), Progesterone Receptor (PR) and HER2 receptors are three important biomarkers routinely used in clinics [25], and are often important in treatment decision. [25]. ER is the most commonly overexpressed receptor and is known to be a strong predictor of response to endocrine therapy such as tamoxifen and the aromatase inhibitors [26]. Breast cancer cells that lack of all three receptors often called Triple negative breast cancer (TNBC) account for 15-20% of breast cancers, but are often much more aggressive in their phenotype [27].

1.2.2 Endocrine therapy in Breast cancer

Approximately 70% of all breast cancer overexpress the estrogen receptor and are known as "ER positive" [28]. In such tumours hormonal treatment is directed at ER signalling pathways, inhibiting the hormonal stimulation of malignant cells by estrogen. Current options for reducing estrogen stimulation of the ER include surgical or chemical ovarian ablation, selective oestrogen receptor modulators (SERMs) and aromatase inhibitors (AIs) [29]. AIs chemically inhibit the production of estrogen by inhibiting the function of the enzyme responsible for the conversion of androgens to estrogen [30, 31] and SERMS such as tamoxifen, fulvestrant and raloxifene inhibit estrogen and ER interactions [32-34].

1.2.3 SERMs and Aromatase inhibitors

Tamoxifen has been responsible for a significant improvement in the life expectancy of many breast cancer patients. It is an anti-estrogen that competitively inhibits the binding of estrogen to ERα, hence downregulating estrogenic signalling within the cell. It is used routinely in clinical practice in the management of patients with both advanced ER positive breast cancer and in the adjuvant (post-operative) treatment of early ER positive cancers [35, 36]. Since its discovery in the 1950s, tamoxifen has become one in a class of drugs called selective ER modulators (SERMs) (Fig. 2A), which now also includes raloxifene, fulvestrant, arzoxifene and lasofoxifene [37, 38].

SERMs act either by targeting ERα for proteasomal degradation or preventing receptor-ligand interaction thereby inhibiting ERα-dependent proliferation [39, 40].

Aromatase Inhibitors (AIs) are a newer class of drug, often used in post-menopausal breast cancer patients, who produce most of their estrogen in the adrenal glands and fat cells and not the ovaries. The mechanism of action of AIs differs from SERMS in that they inhibit the enzyme aromatase, which is responsible for catalysing androgen to estrogen. In doing so, they reduce the amount of circulating estrogen and hence limit estrogen-ER interactions and consequent downstream signalling [41-43]. Some AIs, such as letrozole (Fig 2B) and anastrozol, compete with estrogen precursors for the enzymatic active site of aromatase [44, 45] whereas the AI exemestane (Fig. 2B) covalently binds to aromatase and suppresses its activity [46]. Although hormonal therapies have significantly changed the prognosis of some early breast cancers, many of these tumours will eventually relapse and patients will die from their disease. Cancer cells become resistant to therapies, and patients develop side effects of the medications, hence the need for new treatment options continues.

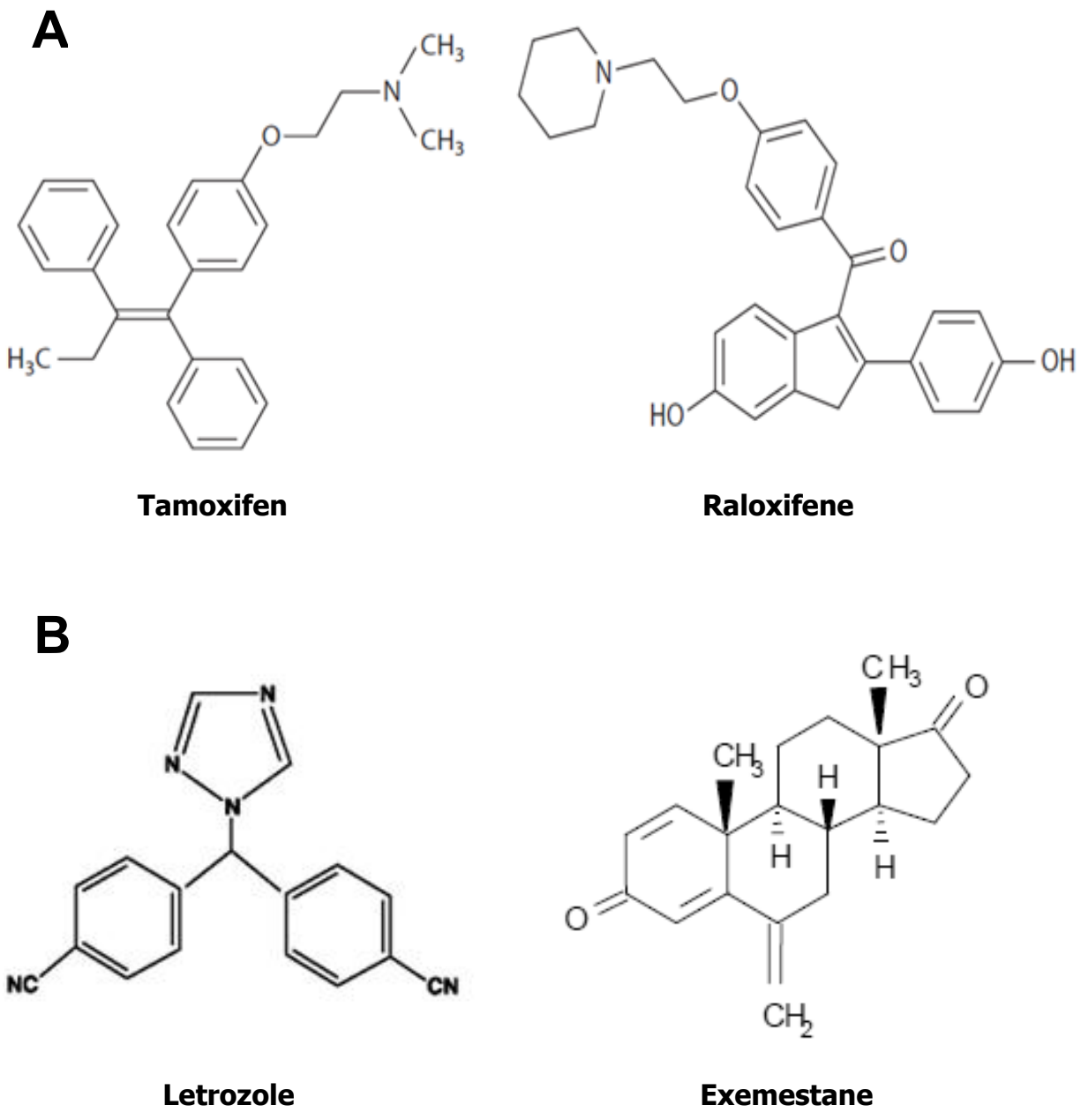
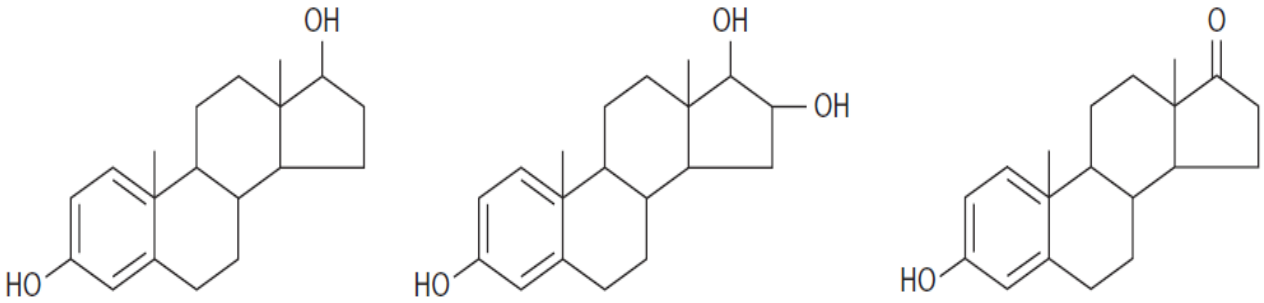


Figure 2: Chemical structure of SERMs and Aromatase Inhibitors. Structures of two common (A) SERMs and (B) Aromatase Inhibitors which are often used in clinical practice. Adapted from [47, 48]

1.3 Estrogens

Estrogen exhibits a broad spectrum of biological functions ranging from brain development, bone health, development of reproductive organs, effects on vascular systems, female sexual behaviour, breast cancer genesis and progression [47, 49, 50]. The most potent intracellular estrogen produced in the body is 17β-estradiol (E2) (Fig 3). Estrone (E1) and estriol (E3) (Fig 3) are also high-affinity estrogen ligands but they are much weaker agonists for the estrogen receptor (ERs) than E2 [51, 52].



Estradiol **Estriol** **Estrone**

Figure 3: Chemical structure of Estrogens. The predominant intracellular estrogen produced in the body is 17β-estradiol (E2) and weaker agonists on estrogen receptors; estrone (E1) and estriol (E3). Adapted from [47]

1.3.1 Estrogen receptor

The effects of estrogen are mediated by two estrogen receptors (ERs), ERα and ERβ, members of the nuclear receptor (NR) gene superfamily. In late 1950s, the pioneering finding in the field was the discovery of estrogen binding protein, today recognised as ERα [53]. Estrogen receptor alpha (ERα) is an important nuclear receptor linked to progression of the majority of human breast cancers [29, 54, 55].

1.3.2 Structure of Estrogen Receptor-α

The human Estrogen Receptor-α (ERα) gene is located on chromosome 6q25.1 and encodes a protein of 595 amino acids. It contains a central DNA binding domain (DBD) consisting of two zinc finger motifs which is involved in DNA recognition and binding, whereas ligand binding occurs in the COOH-terminal multifunctional ligand binding domain (LBD) (Fig 4). The NH2-terminal domain is not conserved and represents the most variable domain both in sequence and length [51]. In the region D, a "hinge" region is involved in nuclear localisation and interaction with heat shock protein complexes [56]. Transcriptional activation occurs through at least two distinct transactivation domains located in the NH2-terminal A/B region (AF-1) and the COOH-terminal E region of the receptor (AF-2) (Fig 4). The AF-1 domain is hormone-independent, whereas the AF-2 domain is estrogen-dependent; both AF domains are required for maximal ERα transcriptional activity [57-60]. The two ERs (ERα and ERβ) share a high degree of sequence homology except in their amino-terminal domains, and they have similar affinities for E2 and bind the same DNA response elements [51].

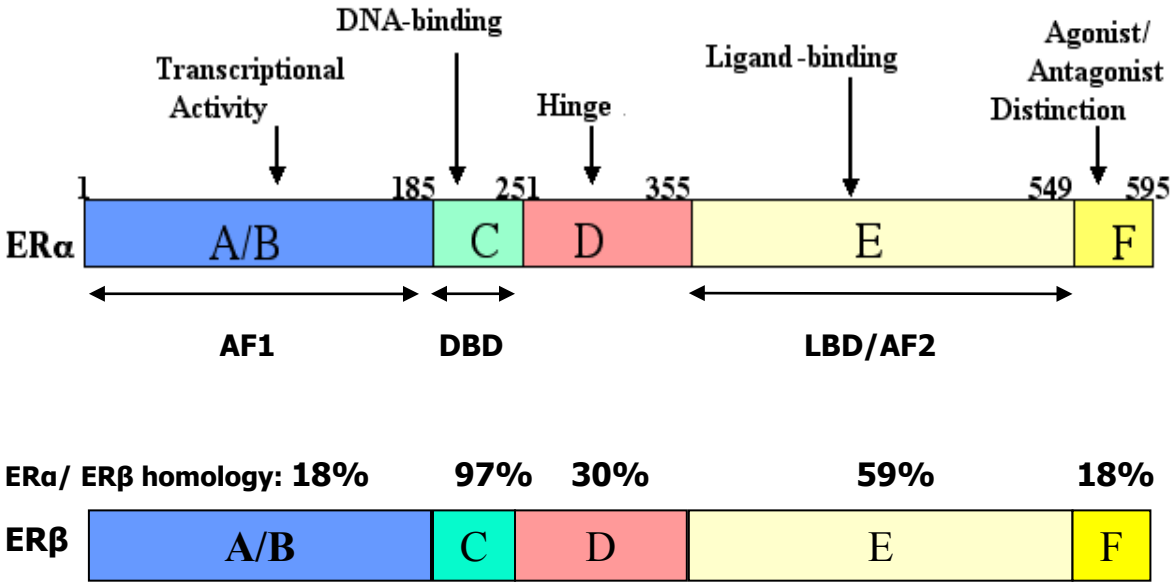


Figure 4: Structure of human Estrogen receptor with different domains. The NH_2 terminal consists of the A/B domain which encodes ligand independent transcription activation function AF1; the C domain contains the DNA binding domain (DBD) while D domain encodes the "hinge" region and finally domain E constitutes the ligand-binding domain (LBD). Adapted from [61]

1.3.3 Estrogen Receptor Signalling

ER mediates expression of its target gene signalling through two different mechanisms; genomic and non-genomic signalling. In genomic signalling, ERs control the expression of genes either directly interacting with DNA via ERE sequences or indirectly through interaction with other transcription factors like c-fos/ c-jun without directly binding to DNA [62]. In the non-genomic signalling pathway, effects of the ER are facilitated by membrane bound ERs without affecting transcription [63].

1.3.4 Genomic signalling of Estrogen Receptor

Genomic functions of ERα are mediated by either classical or non-classical signalling. Ligand-receptor binding of estradiol and ERα mediates gene expression by causing ERα binding to a 13bp palindromic sequence called the 'Estrogen Response Element' (ERE). Classical signalling of ERα involves direct interaction with DNA via ERE sequences in promoter regions of estrogen-regulated genes with the help of co-activators (Fig 5). EREs were first identified in the *Xenopus* vitellogenin A2 gene and found to have the consensus sequence AGGTCAnnnTGACCT, where 'n' represents any nucleotide [64].

ERα regulated genes, including pS2/TFF1 (trefoil factor 1), were first identified by cloning of cDNAs induced by estrogen treatment of breast cancer cells [65]. Upon 17-β-estradiol (E2) binding, ERs mediate transcription with the help of co-activators like SRC-1, TIF2 and AIB1 by interacting directly with specific response elements (EREs) located in the promoter region of its target genes (Fig 5) [66-68]. AIB1 (also known as SRC-3), is a p160 co-activating oncogene

overexpressed in different types of cancer, specifically breast tumours [69] and is associated with regulating ERα transcriptional activity [70]. AIB1 has been shown to increase ERα transcriptional activity through chromatin remodelling by recruiting co-factors that possess histone acetyl-transferase activity [71]. Remarkably, it has also been shown that AIB1 regulates ERα degradation by the recruitment of components involved in the ubiquitin-proteasome pathway. However, suppressing AIB1 leads to ERα stabilisation in the presence of estradiol (E2) and subsequent reduction of ERα transcriptional activity [1, 72]. The activity of AIB1 is regulated by phosphorylation which leads to high level of AIB1 degradation [73, 74]. After estrogenic induction, ERα mRNA stability is substantially reduced within 1 hour of stimulation [58, 75]. Furthermore, the interactions between E2 and ERα speed-up receptor degradation through the ubiquitin-proteasome pathway, an effect associated with its major co-activator AIB1 [58, 76].

Through the non-classical pathway, ERα can also regulate gene transcription indirectly by binding to nuclear proteins, such as AP1 and SP1 transcription factors [77]. Estrogen bound ER can initiate expression of collaginase and IGF-1 through an interaction with c-fos and c-jun at AP1 binding sites, and stimulation of CyclinD1 is through the interaction with SP1 [63]. The cellular response to estrogen is highly regulated at multiple levels including transcription, RNA stability and post-translational modifications [75, 76, 78, 79].

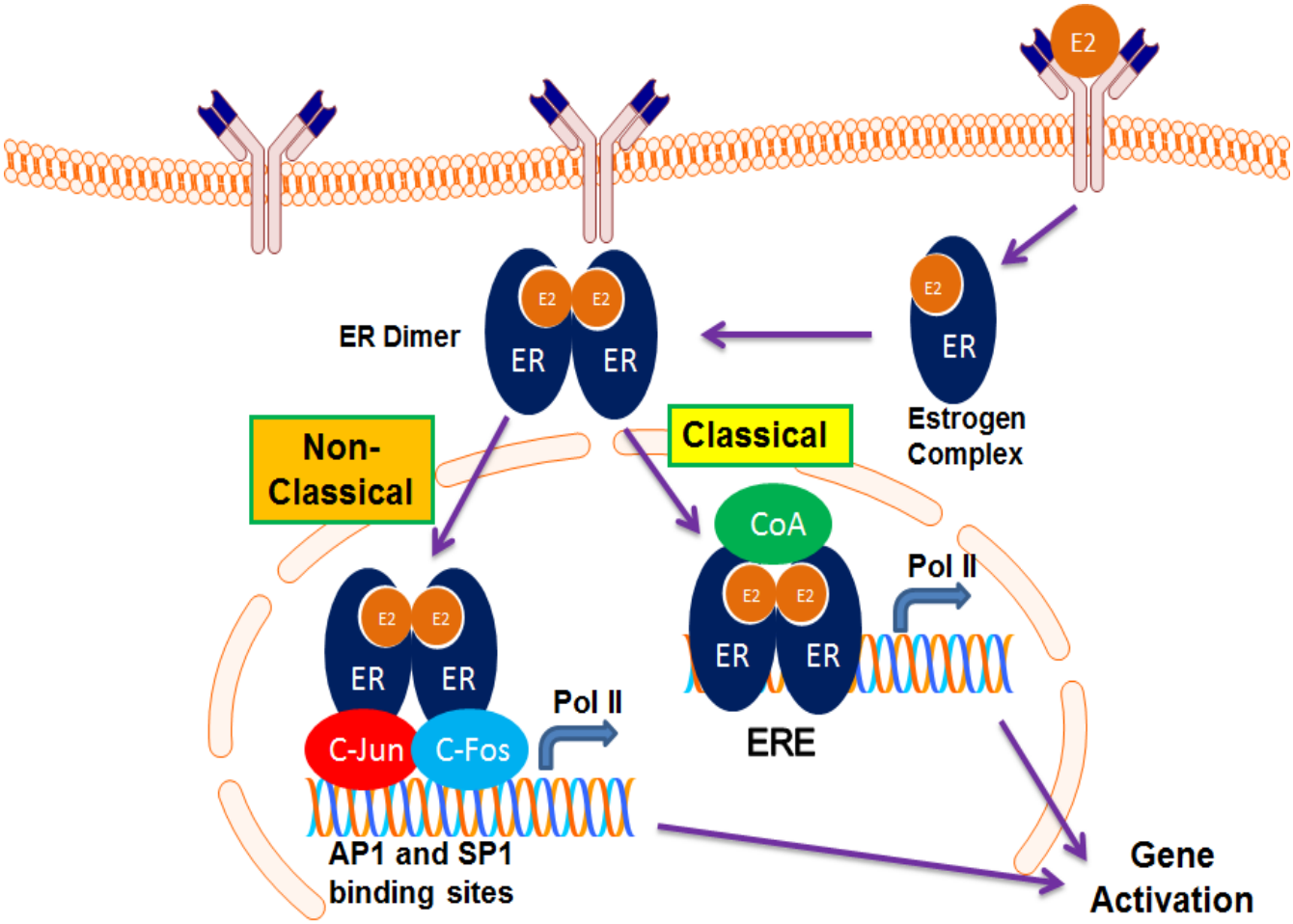


Figure 5: Model of classical and non-classical actions of the estrogen receptor (ER). In Classical pathway, Estrogen binds to ER and translocates to the nucleus where activated the receptor binds to ERE and recruits co-activators to activate gene transcription. In non-classical pathway, the activated receptor binds to transcription factors like c-jun/c-fos and cyclinD1 via AP1 and SP1 sites respectively and thereby enhance transcription of the target genes. Modified from [80].

After ER ligand activation, both genomic and non-genomic ER pathways regulate different growth factor signalling pathways that are crucial for breast cancer biology. The selective modulation of ER pathways, in combination with inhibition of upstream and downstream activated growth factor signalling pathways, will lead to more effective treatments [81-83]. There are several ongoing randomized clinical trials currently hoping to improve response rates in patients who might benefit from such combined treatments. The significant treatment of breast cancer will also require a combination of basic experimental models with genomic and proteomic tumour characterization [39, 84, 85].

Over the last decade RNAi biology has completely changed the understanding of gene regulation. Small non-coding RNAs like microRNAs (mRNAs) have already proven their role in cancer biology and other diseases and shown their importance at different levels of gene regulation. The role of mRNAs in the regulation of the estrogen receptor pathway is likely to further advance our understanding of this important and clinically relevant cell signalling pathway, and could result in the identification of novel drug targets, and a new approach to the prevention and treatment of human breast cancer.

1.4 MicroRNAs

MicroRNAs (miRNAs) are endogenous non-coding RNAs of approximately 22 nucleotides length which were first discovered in *Caenorhabditis elegans* in 1993 [99, 100], and are now known to be important regulators of gene expression, involved in the control of many biological processes including cell differentiation, growth and metabolism [95-98]. They are one of the largest gene families comprising of ~1% of the genome. Through their base pair interactions with messenger RNA (mRNA) they act as post-transcriptional modulators of gene expression, preventing translation of mRNA and resulting in post-transcriptional gene repression [88-90]. Base pairing at the 3' UTR end, 5' UTR end, promoter or coding regions of the mRNA does not need to be fully complementary to induce repression [91, 92].

In other circumstances, when the miRNAs interact partially with the sequences of their target mRNAs called seed region, they induce their translational repression

Most human miRNAs are predicted to interact with at least several hundred target mRNAs in a sequence specific manner involving Watson & Crick base-pairing within 2-8 nucleotides of the miRNA [88, 93, 94]. A single miRNA can bind to and regulate many different mRNA targets and, conversely, several different miRNAs can bind to and cooperatively control a single mRNA target [101, 102]. Analogous to transcription factors, miRNAs regulate mRNAs in a combinatorial fashion and single miRNAs can repress the translation of many mRNAs [103].

1.4.1 Functions of MicroRNAs

Over the past decade, miRNAs have proved to have an important role in most biological pathways and also to play a critical role in many human diseases [86]. Hence an understanding of their function is imperative if we are to better understand disease.

Initially it was thought that microRNAs targeted the 3' UTR of mRNA genes and down regulated them either by degrading the mRNA or by inhibiting their translation in the cytoplasm [87-89]. However, in more recent years, it has been shown that miRNAs can localize in the nucleus of cells [90] and that they can also target other regions of the genes including the 5' UTR, promoter regions and coding regions [91-94]. As described below, miRNAs have also been shown to up-regulate translation [92, 95].

1.4.2 Distinct role of MicroRNAs

Eiring *et al*. [96] showed for the first time that miRNAs are also able to act as decoys (dual role of miRNAs) and in doing so prevent translational repression. The study showed that miR-328 acts as a decoy by binding to hnRNPE2 (Heterogeneous nuclear ribonucleoprotein E2) which subsequently prevents it from repressing CEBPA mRNA which is involved in myeloid cell differentiation. It has also been recently shown that miR-29 prevents HuR from binding to the 3'UTR of the A20 transcript, therefore acting as a decoy for HuR and shielding the A20 trancripts [97]. Pseudogenes such as PTENP1 behave in a similar manner, but instead act as decoys for the miRNAs themselves. For example,

PTEN1P binds to oncogenic miRNAS (onco-miRs), which prevents them from binding to the PTEN transcript, an important tumour suppressor gene [98].

Another important role recently identified is that of competing endogenous RNAs (ceRNAs). It has been shown that ceRNAs can regulate miRNA through their ability to compete for miRNA binding on the target gene, acting as endogenous decoys for miRNAs [99]. Such active regulation of miRNAs may be important in cancer biology as well as playing a pivotal role in drug resistance, and is worthy of further exploration [100]. It has been shown that mir-369-3 targets the AU-rich elements of the TNF gene, where most of RNA-binding proteins bind [95]. miR-373 has been shown to bind to the promoter of E-cadherin (CDH1) and regulate transcription, which shows the role of miRNAs at transcriptional level [92].

miRNAs are also important in epigenetics. These "epi-miRNAs", such as the recently identified miR-29 family, directly control epigenetic machinery through a regulatory loop in this case of the miR-29s by targeting the regulating enzymes DNMT3A and DNMT3B in lung cancer [101]. Other epi-miRNAs have been shown to control the expression of regulatory enzymes of the epigenetic machinery [102-106]. There are more distinct roles of miRNAs discovered over the recent years which have been summarized in the figure below (Fig. 6). The field of miRNA research therefore requires further focus on the interplay that connects the regulatory mechanisms and their functions.

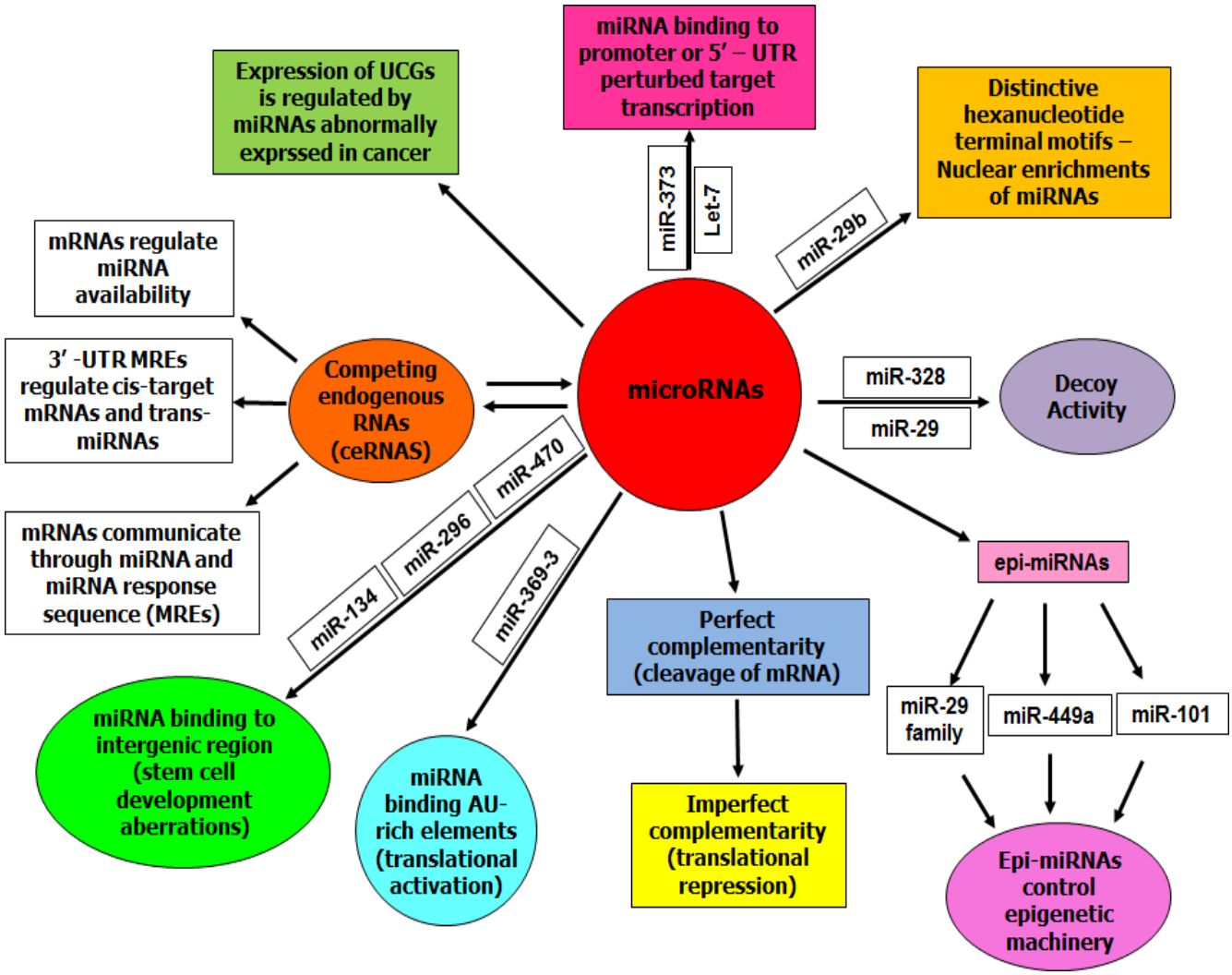


Figure 6: Cross talk between miRNAs and their targets. Interactions between miRNA and their targets through various mechanisms. These are the new functions/role of microRNAs discovered over the recent years. Modified from [87].

1.4.3 miRNA genes

miRNA genes were initially thought to be transcribed from the intergenic regions (between protein-coding genes) of the genome but later it was also shown most of them are located within the intron or exon regions of the protein-coding genes [107-110]. Most of the miRNAs are transcribed from a single polycistronic transcription units (TU) and are structured into clusters; more than half of the miRNA loci are in close proximity (<50kb) to the next miRNA [111-113]. Clusters often contain two or more miRNAs with similar sequences and this combination allows synergistic biological effects [111].

1.5 MicroRNA Biogenesis

miRNA biogenesis was initially thought to be universal for the processing of all mature miRNAs, but since their discovery many alternative processing pathways have been discovered [114].

1.5.1 Trancription of the primary miRNA transcript and DROSHA processing

In a typical 'canonical pathway' most of the miRNAs are transcribed from endogenous genes by RNA polymerase II [115, 116] or in some cases by RNA polymerase III [117], then the transcripts are capped and polyadenylated [118]. The resulting primary, or pri-miRNA, transcript extends both 5′ and 3′ from the miRNA sequence, and two sequential processing reactions trim the transcript into the mature miRNA.

In the first processing step, which occurs in the nucleus, the pri-miRNA is cleaved (also called cropping) into pre-miRNA, an approximately 70-nucleotide hairpin stem loop intermediate, by a microprocessor complex which consists of Drosha, an RNase III enzyme and DGCR8 which functions as a molecular ruler to govern the accurate cleavage site [109, 119, 120]. Over the years it is been discovered that the microprocessor complex contains a diversity of cofactors including the hnRNPs, RNA helicases p68 (DDX5) and p72 (DDX17), DEAD box and many other RNA-binding proteins [121, 122]. Further discussion of the RNA binding proteins involved in Drosha processing is found below. These assisting factors may function to promote the fidelity, specificity or activity of Drosha cleavage [118]. Surprisingly, Drosha-mediated processing of pri-miRNAs into pre-miRNAs is not always compulsory. A few intron-derived miRNAs called "mirtrons" are directly released from their host transcripts after splicing as pre-miRNA, thereby skipping the Drosha cleavage [123-125].

1.5.2 Nuclear transport of the pre-miRNAs by Exportin-5 (xpo-5)

After its formation, the pre-miRNA is actively transported from the nucleus to the cytoplasm by the Ran-GTP-dependent transporter Exportin 5 (Exp 5) [126, 127]. Drosha cleavage occurs co-transcriptionally, before splicing of the host RNA [109] and produces a product with a two-nucleotide 3' overhang, which is a distinguishing characteristic of RNase III-mediated cleavage. The overhang is recognised by XPO-5, which transports the pre-miRNA into the cytoplasm via a Ran-GTP-dependent mechanism [113, 128]. When there is depletion of XPO-5, there is accumulation of the pre-miRNAs, and mature miRNAs are significantly

reduced [127]. XPO-5 together with complex [guanosine triphosphate (GTP) bound of the cofactor Ran GTPase in the nucleus], binds to the precursors and then releases the precursors in the cytoplasm following the hydrolysis of Ran-GTP to Ran-GDP [127, 129].

1.5.3 Cytoplasmic processing of pre-miRNAs by Dicer

In the cytoplasm, the pre-miRNA is further 'diced' into an approximately 22-nucleotide miRNA duplex by Dicer, another RNase III enzyme, in collaboration with the dsRBD proteins TRBP/PACT [130, 131]. Dicer was originally found to have a role in generating siRNAs for RNA interference (RNAi) but later it was shown that removal of Dicer eliminates the production of mature miRNAs and therefore has a crucial role for miRNA processing [132]. Dicer cleaves the precursors into 22nt miRNA duplexes by recognising the two-nulceotide overhangs at the 3' end. In some cases, like the processing of let-7 family (which is negatively regulated by Lin-28), this is done by recruiting terminal uridylyl transferases (TUTs) to the pre-miRNA and producing a long single-stranded tail of Uracils (known as oligo-uridylation) at the 3' end of the precursor, therefore blocking further processing by Dicer [133]. However, recently it was reported that pre-let-7 can be uridylated by TUTs (TUT2, TUT4 and TUT7) even in the absence of LIN28, but in this case only one Uracil (mono-uridylation) is added, producing a two-nucleotide 3' overhang which is recognised by Dicer for further processing [134]. Dicer interacts with its partners TRBP and PACT, neither of which is required for the cleavage reaction itself but contribute to the formation

of the RNA-induced silencing complex (RISC) by recruiting to Ago2, the final core component [130, 135, 136].

1.5.4 Loading of miRNAs to the RISC complex for mRNA tageting

The major components of RISC are the proteins of the Argonaute (AGO) family which function in both miRNA and siRNA pathways. Ago2 is the most crucial component of the RNA-induced silencing complex (RISC) [137]. The miRNA/miRNA* duplex is unwound by universal helicase and separated into a functional guide strand which is complementary to the mRNA target, and a passenger strand which is subsequently degraded [107]. However the universal helicase responsible for miRNA/miRNA* unwinding has not yet been identified [114]. The functional guide strand selected for incorporation into the miRISC and guiding depends on the thermodynamic stability of the base pairs at the two ends of the duplex; the miRNA strand with the relatively unstable base pair at its 5' end (e.g. GU pair compared with a GC pair) is loaded into miRISC [113, 114, 138, 139]. The miRISC then binds by imperfect base pairing to the 3'UTR of the target mRNA in a sequence-specific manner which is called the ‘seed region’, and induces mRNA cleavage or translational repression [140].

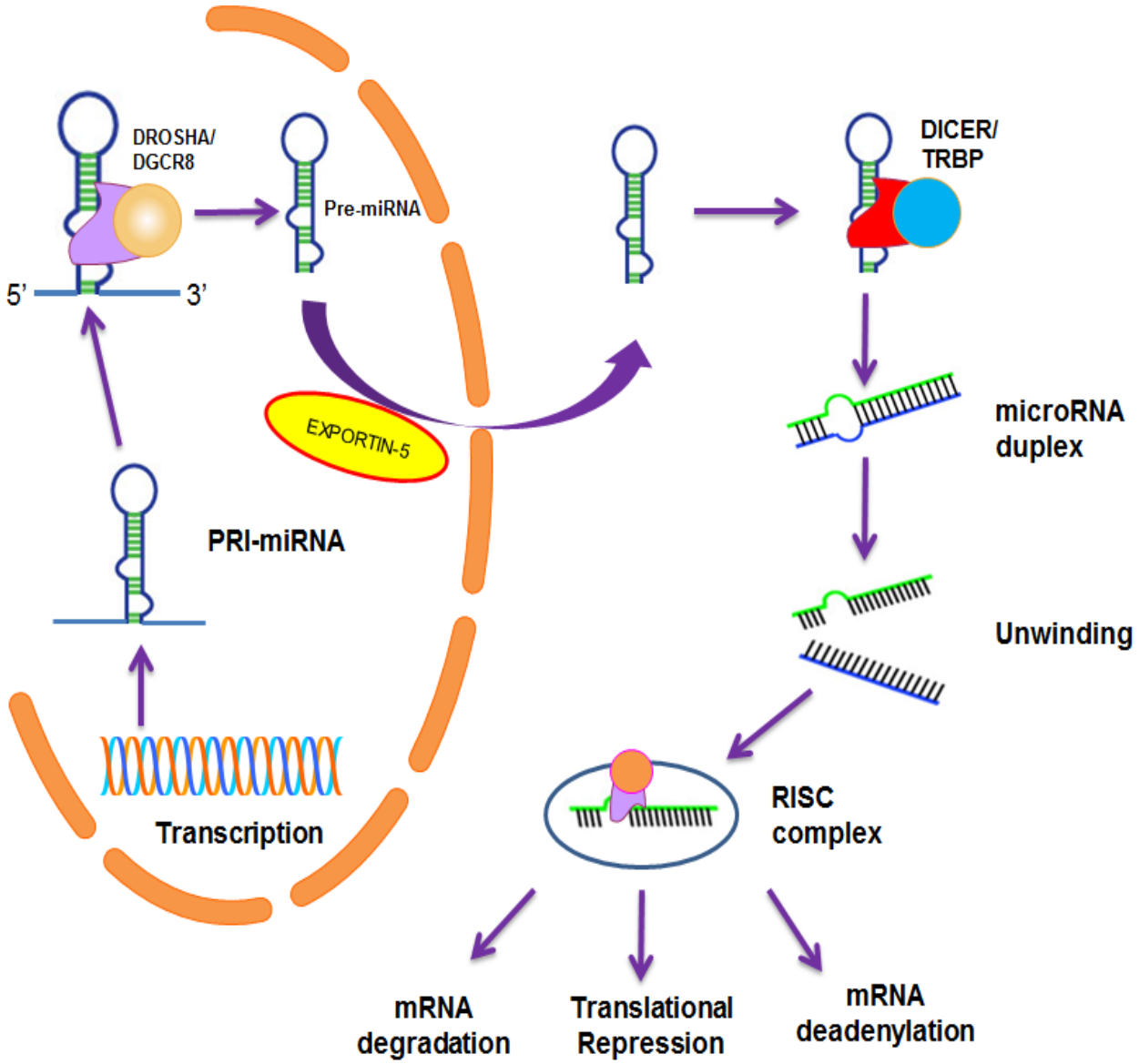


Figure 7: The 'linear' canonical pathway of microRNA processing. The miRNA processing pathway has long been viewed as linear and universal to most of the mammalian miRNAs. Modified from [141]

1.6 microRNA and cancer

MicroRNAs are involved in a wide range of developmental and physiological processes; their dysregulation is linked to many human cancers [86]. Interestingly, it was found that more than 50% of miRNA genes are located in cancer-associated genomic regions or in fragile sites (deletions or amplifications) [142]. Over the years, many studies have shown that miRNAs can act as oncogenes or tumour suppressor genes, creating molecular signatures for a variety of cancers [143-145]. miRNAs have shown to be diagnostic and prognostic markers for human cancers, as well as targets for molecular therapy [146]. Table 1 shows a timeline of the most important discoveries linking miRNA's role in cancer, since their discovery in 1993.

Year	Discovery	Species	Reference
1993	first miRNA (lin-4) discovered	*C. elegans*	[147, 148]
2002	First miRNAs alterations (miR15 and miR16 deleted or downregulated) found in in most chronic lymphocytic leukemias	Human	[149]
2004	miRNA genes (>50%) are located in cancer-associated genomic regions or fragile sites	Human	[142]
2004	First miRNA (let-7) described as diagnostic/prognostic biomarker	Human	[150]
2005	miRNA-target interaction relevant to cancer (by dowregulating RAS)	*C. elegans,* Human	[151]
2005	First microRNA cluster (*mir-17–92*) to describe as a potential human oncogene	Human	[152]
2005	First time to link between miRNAs and the MYC oncogene	Human, rat	[153]

2006	MicroRNAs (hsa-mir-155 and hsa-let-7a-2) as molecular signatures that differ in tumor histology	Human	[154]
2006	Epigenetic regulation (DNA methylation and histone deacetylase inhibition) of miRNAs that may act as tumor suppressors.	Human	[155]
2007	First miRNA (mir-10b) in deregulation of cancer metastasis	Human	[156]
2007	miRNAs can affect epigenetic changes and cause the reactivation of silenced tumor suppressor genes	Human	[101]
2007	Some miRNAs can target 5′-UTR of the genes.	*C. elegans,* Human	[91]
2007	miRNAs can regulate ncRNAs from the category of long ultraconserved genes (UCGs)	Human	[157]
2008	functional single nucleotide polymorphism (SNP) in the miRNA seed region	Human	[158]
2009	proof of concept of miRNA delivery (mir-26a) as cancer therapy in HCC.	Human, Mouse	[159]
2010	miRNA (mir-328) as molecular decoys	Human, Mouse	[160]
2010	overexpression of a single miRNA (mir-21) is sufficient to cause cancer	Mouse	[161]
2011	Use of Engineered biological systems (HeLa cancer cell classifier selectively identifies HeLa cells and triggers apotosis without affecting non-HeLa cell types based on expression levels of a customizable set of	Human	[162]

	endogenous microRNAs)		
2013	miRNAs act as modulators of mRNA–mRNA interactions	Human	[163]
2013	A Phase I study of MRX34, a miR-34-based therapy and the first ever microRNA mimic to advance into a human clinical trial	Human	[164]

Table 1: Timeline of important discoveries of miRNA's role in cancer.

Modified from [87]

1.6.1 miR-17-92 Cluster in Cancer

The miR-17-92 cluster, a polycistronic gene, was investigated following a series of observations linking the miRNAs arising from this cluster to cancer pathogenesis [165]. The human mir-17-92 cluster is located within 800 base pairs in the noncoding gene C13orf25 with a chromosomal location at 13q31.3. It encodes for six miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1 [86]. Based on these observations, it was shown for the first time by direct experimental evidence that miR-17-92 cluster has oncogenic activity [152]. Ancient replications have given rise to two mir-17-92 paralogues in mammals: mir-106b-25 and mir-106a-363. mir-17-92 cluster is undoubtedly the most studied class of miRNAs in cancer.

miRNAs derived from mir-17-92 have shown to be oncogenic in lymphomas, leukemia, thyroid, colonic and lung cancer [152, 166-170], and been shown to act as a tumour suppressors in breast cancer, by down-regulating AIB1 and

Cyclin D1 [171, 172]. In addition, it is been discovered that the genomic 13q31 area, including mir-17-92, is associated with loss of heterozygosity in breast cancer [173].

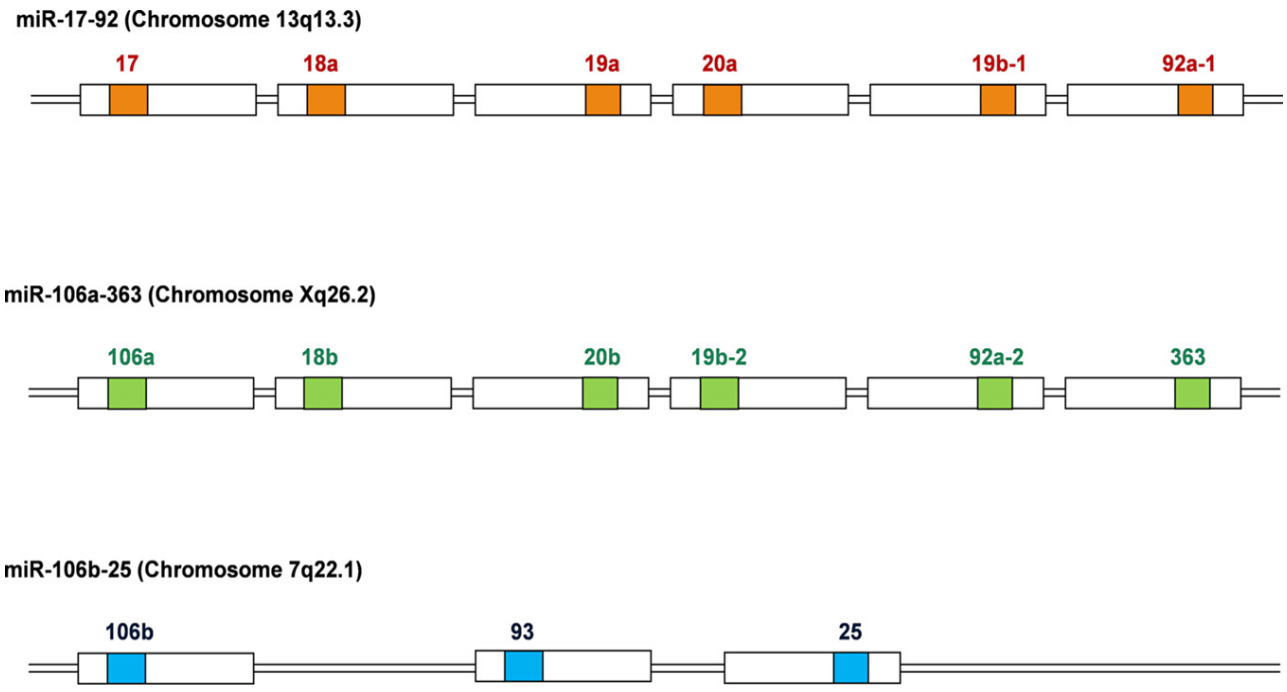


Figure 8: Primary miRNA transcript structures of the 3 paralogous families. Human mir-17–92, mir-106a-363 and mir-106b-25 clusters. Adapted from [1]

The miR-17-92 cluster first came under scrutiny when it was reported that transcription of this cluster is directly transactivated by c-Myc [153], a oncogenic transcription factor that is frequently overactive in cancer cells. These findings provided one of the first critical demonstrations that miRNAs are functionally incorporated into oncogenic pathways and are crucial to cancer development. Several independent bodies of research have further confirmed that the miR-17-92 cluster and its paralogues can act as oncogenes [174-177]. It was also shown that the expression of the paralogue cluster miR-106a-363 is normally extremely low and when ectopically transcribed, the miRNAs from this cluster may act in a similar manner to the miR-17-92 cluster [178]. All these findings suggest the important role of miR-17-92 cluster and its paralogue cluster in cancer biology.

1.6.2 miR-17-92 Cluster in Breast Cancer

Prior to this research, little evidence existed for the role of the mir-17-92 cluster in breast cancer, however, in recent years, many studies have shown that mir-17-92 miRNAs have both an oncogenic and tumour suppressive role in breast cancer. For example, mir-19 has been shown to play an oncogenic role in breast cancer [179-183] whilst mir-18a can act as a tumour suppressor in breast cancer [1, 184, 185]. These findings suggest that the individual miRNAs derived from this cluster can be used as potential therapeutic target in breast cancer.

miR-17	**CAAAGUGCUUACAGUGCAGGUAG**
miR-20a	**UAAAGUGCUUAUAGUGCAGGUAG**
miR-20b	**CAAAGUGCUCAUAGUGCAGGUAG**
miR-106a	**AAAAGUGCUUACAGUGCAGGUAG**
miR-106b	**UAAAGUGCUGACAGUGCAGAU**
miR-93	**CAAAGUGCUCUUCGUGCAGGUAG**
miR-18a	**UAAGGUGCAUCUAGUGCAGAUAG**
miR-18b	**UAAGGUGCAUCUAGUGCAGUUAG**
miR-19a	**UGUGCAAAUCUAUGCAAAACUGA**
miR-19b	**UGUGCAAAUCCAUGCAAAACUGA**
miR-25	**CAUUGCACUUGUCCUCGGUCUGA**
miR-92a	**UAUUGCACUUGUCCCGGCCUGU**
miR-363	**AAUUGCACGGUAUCCAUCUGUA**

Figure 9: MicroRNAs derived from miR-17-92 cluster and its paralogue clusters (mir-106a-363 and mir-106b-25). The seed sequences are highlighted in red - the regions considered most important for target selection. miRNAs of these clusters can be grouped into four families: the miR-17 family (miR-17, miR-20a/b, miR-106a/b, and miR-93); the miR-18 family (miR-18a/b); the miR-19 family (miR-19a/b); and the miR-25 family (miR-25, miR-92a, and miR-363). Adapted from [178].

1.7 RNA binding proteins

RBPs play an important role in most aspects of RNA biology, transcription, pre-mRNA splicing, mRNA export, translation and decay [186, 187]. They have also been shown to have a role in post-transcriptional modifications like polyadenylation and RNA editing [188-190]. Generally RNAs in cells have dynamic interactions with RNA-binding proteins (RBPs) to form ribonucleoprotein (RNP) Complexes [191]. Post-translational modifications are important for the stability of the proteins [192]. These modifications of RBPs cause additional layers of complexity, as they can alter the RNA-binding, function and localization of the RNP [188]. The three common types of modifications reported for RBPs that can change RNA binding are phosphorylation, arginine methylation and small ubiquitin-like modification (SUMO) [193-195]. For RNA-protein interactions, the sequence of the RNA target is very important and also the protein-protein interactions are crucial factors in shaping the formation of an RNP. Also, more than one RBP can bind to a specific sequence of the target RNA [196].

One of the important RBPs are heterogeneous nuclear ribonucleoproteins (hnRNPs) which are mostly present in the nucleus, involved in gene transcription and also in post-transcriptional modification of the newly synthesized pre-mRNA [197]. There are so many types of hnRNPs, and most of them tend to share the same type of RNA binding domain [198]. Some of the well-characterized RNA-binding domains include the following: RNA binding domain, RNA recognition motif, K-homology (KH) domain (type I and type II), RGG (Arg-Gly-Gly) box, Sm domain, DEAD/DEAH box, zinc finger (ZnF), double stranded RNA-binding

domain (dsRBD), cold-shock domain, Pumilio/FBF (PUF or Pum-HD) domain and the Piwi/Argonaute/Zwille (PAZ) domain [188]. RBPs often contain multiple RNA-binding domains and the functions of the RBPs are generally characterized by the presence these domains [199].

1.8 MicroRNA regulation by RNA binding proteins

The regulation of miRNA expression occurs at three levels: pre-transcriptional, transcriptional, and post-transcriptional [107, 114, 139, 200]. Most of the microRNA regulation by RBPs occurs at the post-transcriptional level [201, 202]. Maturation of miRNAs requires coordinated post-transcriptional processing mechanisms where DROSHA crops pri-miRNA transcripts to pre-miRNA hairpin structure and further DICER cleaves pre-miRNA to form mature miRNAs [203]. Apart from DROSHA and DICER being the main component of the microprocessor complex there have been several RBPs which are important for miRNA processing [204]. RBPs like DGCR8 and TRBP regulates the global miRNA processing; but there are also RBPs involved in processing of individual miRNA or subset of miRNAs. miR-18a is derived from pri-miR-17-92 transcript which also encodes for five other miRNAs (Fig. 8). Initially, it was shown that hnRNP A1, an mRNA splicing regulator, stimulates DROSHA processing of miR-18a from the miR-17-92 cluster. pri-miR-18a had two regions similar to the consensus hnRNP A1-binding site, UAGGGA/U, within its terminal loop and stem but these consensus site were not present with the other miRNAs of the cluster [205, 206]. The specific interaction of hnRNPA1 with pri-miR-18a leads to structural

rearrangement of the hairpin to produce a more favourable Drosha/DGCR8 binding and cleavage site [206]. Therefore hnRNPA1 acts as a chaperone for specific cropping of pri-miR-18a by Drosha/DGCR8. Interestingly, further studies showed that approximately 14% of human miRNAs have highly conserved loop sequences, suggesting the regulation of miRNA processing by hnRNPA1 might extend well beyond miR-18a [206]. Lin-28 was the first RBP discovered to be a negative regulator of microRNA processing both at DROSHA and DICER processing. Lin-28 inhibited microprocessor-mediated cleavage of let-7 family in the nucleus [207], by competing with DROSHA [208, 209]. Further studies revealed Lin-28 also inhibited DICER-mediated cleavage of the pre-let-7 family in the cytoplasm [210] by recruiting a terminal uridylyl transferase (TUTase) to the precursor and thereby inhibiting the processing. Another important RBP regulating miRNA processing was the KH type splicing regulator protein (KSRP) which recognizes the G-rich regions (particularly GGG triplet) present within the terminal loops of a subset of pri-miRNAs, thereby promoting DROSHA processing [211]. KSRP was also shown to interact with the terminal loop of the miRNA precursors during nucleo-cytoplasmic transfer and thereby promoting the DICER processing of the target pre-miRNAs in the cytoplasm. This suggests that the sequence specific recognition by the RBPs is important to extend regulation of miRNA processing of the individual miRNAs. Recent studies also revealed the binding of RBPs to specific sequences in the terminal loop regions of miRNA precursors either competes for binding or stimulates recruitment of processing factors [212]. The table below shows all the RBPs known to regulate the microRNA processing till date.

Protein	Motifs	Known Activity	Mechanisms	Target miRNA	Referen-ces
DGCR8	dsRBD	Binding to Drosha	Stabilizing Drosha	Global	[118]
TRBP	dsRBD	Binding to Dicer, MKK phosphorylation site	Stabilizing Dicer	Global	[213]
p53	DNA binding	Tumour Suppressor	Binding to p68 and Promoting Drosha cleavage	miR-16-1, miR-143	[214]
SMADs	DNA binding	Signal transducers of TGFβ	Binding to p68 and Promoting Drosha cleavage	miR-21, miR-199a	[215]
ERα	DNA binding	Nuclear Receptor	Binding to p68/p72 and inhibiting Drosha cleavage	A subset	[216]
hnRNP A1	RRM, M9	Pre-mRNA splicing	Chaperone for Drosha/DGCR 8 binding	miR-18a	[205]
KSRP	KH	mRNA decay	Promoting Drosha and Dicer processing	A subset	[211]
ARS2	Plant SERRATE homolog	Nuclear Cap-binding	Enhancing Drosha Processing	Global	[217, 218]
Exportin-5	RanBP	Binding to tRNAs and pre-miRNAs	Nuclear transport of pre-miRNA	Global	[219, 220]
LIN-28	CCHC-type zinc finger	Promoting pluripotency	Inhibition of Drosha and Dicer processing, and Recruiting TUT4	let-7	[133, 208, 221] [210] [207]

TUT4	Poly(A) polymerase, CCHC-type zinc finger	Terminal uridylation	Binding to LIN-28 and inhibiting Dicer processing	let-7	[213, 221, 222]
Mei-P26	TRIM-NHL (RING finger)	Ubiquitinylation	Binding to miRISC and inhibiting miRNA activity	A subset	[223]
Argonautes	PAZ, PIWI	Components of RISC	Stabilizing associated miRNAs	Global	[220]
SF2/ASF	RNA recognition motif (RRM)	SR protein splicing factor	binds directly to pri-miR-7 and promote its nuclear cropping.	miR-7, miR-29b, miR-221, and miR-222)	[224]
ADARs	dsRBD	A-I RNA editing	Inhibition of Drosha and Dicer processing	A subset	[225, 226]
p68/p72	DEAD-box	Components of Microprocessor	Promoting Drosha Cleavage	A subset	[122]
XRN-2	5′ to 3′ exoribonuclease	Exoribonuclease	Degrading miRNA	Global	[227]
GLD2	Poly(A) polymerase	Terminal adenylation	Stabilizing miRNA	miR-122	[228]
mLin41	TRIM-NHL (RING finger)	Ubiquitinylation	Binding to Ago2 and targeting it for degradation	Let-7 and others in ES cells	[229]
NHL-2	TRIM-NHL (RING finger)	Ubiquitinylation	Binding to miRISC and enhancing miRNA activity	A subset	[230]

NF90-NF45	dsRBD	Transcription factor	Binds to the Microprocessor complex (Drosha) and Inhibits the Drosha processing	let-7a	[231]
AUF1	RNA binding domain (RBD)	RNA binding protein	Suppresses the Dicer expression and blocks Dicer processing	Global	[232]
PTEN	Phosphatase domain, C2	Tumour Suppressor	Inhibits the Drosha processing indirectly by down regulating RNH1	miR-21	[233]
MBNL1	Zinc finger domain	Pre-mRNA splicing	Positively regulates Dicer processing	miR-1	[234]
SNIP1	Forkhead-associated (FHA) domain	Involved in TGF-β and NF-KB signalling pathways	Inhibits the Drosha processing	Global	[235]
BRCA1	RING finger	E3 ubiquitin ligase	Positively regulates Drosha processing	let-7a-1, miR-16-1, miR-145, miR-34a	[236]
TUT2, TUT4 and TUT7	Zinc Finger CCHC Domain	Terminal uridylation	mono-uridylate group- II pre-miRNAs and regulates dicer processing	Subset	[134]

AR	DNA binding	Nuclear Receptor	Positively regulates Drosha processing of pri-miR-23a27a24-2 cluster.	Subset	[237]
BCDIN3D	DNA binding	Methylation	Negatively regulates dicer processing	miR-145	[238]
MCPIP1	Zinc Finger CCCH Domain	Ribonuclease	suppresses miRNA biogenesis via cleavage of the terminal loops of precursor miRNAs	Global	[239]
TDP-43	DNA binding	DNA and RNA-binding protein	Interacts with Dicer complex and promote processing	Subset	[240]
EGFR	Ligand Binding, Tyrosine kinase domain	Receptor tyrosine kinase binding ligands of the EGF family	Phosphorylate AGO2 in response to hypoxic stress, which in turn reduces the binding of Dicer to AGO2	Subset	[241]
c-Myc	DNA binding	DNA and RNA-binding protein, transcription factor	c-Myc binds directly with drosha and promote processing	Global	[242]
SND1	Staphylococcal Nuclease Domain	Transcriptional coactivator/ Important component of RISC complex	SND1 interacts with the precursors of mir-92a cluster and blocks the processing	miR-92a	[244]

Table 2: Post-transcriptional Regulators in mammalian miRNA Biogenesis. Modified from [243]

It is established that RBPs like Dnd1 or HuR can regulate the function of miRNAs in different ways either by affecting the biogenesis of miRNAs or by directly interacting with the miRNA binding sites, competing with the miRNA binding sites on mRNAs and therefore regulating the miRNA function [245]. A relationship between miRNAs and RBPs clearly exists which is important for proper function of processes involved in differentiation, cell cycle, stress, and cell survival [246]. As miRNAs play a very important role in cancer, and RBPs regulate miRNA biogenesis and activity, it suggests that the interplay of miRNA and RBPs is important for the regulation of cancer pathways.

1.9 Estrogen Receptor Phosphorylation

Phosphorylation is one the most important post-translational modifications and has been shown to regulate many nuclear receptors including Estrogen Receptor. ERα has shown to be phosphorylated in the absence of a ligand as well as when bound to estrogen and anti-estrogens [247]. Functions of some of the important phosphorylation sites have been summarized below (table 3 and fig 10).

Site of Phosphorylation	Function	Kinase (putative)	Reference
Ser46/47	Transcription	PKC	[248]
Tyr52	Transcription, Protein stability, Cell growth/invasion	cABL	[249]
Ser104/106	Transcription, Coactivator binding	Cyclin A/CDK2, ERK1/2, GSK3β	[250-252]
Ser118	Transcription, Coactivator binding, Protein stability, RNA splicing, Cell growth/invasion	CDK7, ERK1/2, IKKα, GSK3β, DNA-PK,	[253-256]
Ser167	DNA binding, Transcription	p90 RSK1, S6 K1, AKT, IKKε, CK2	[257-261]
Tyr219	DNA binding, Dimerization, Transcription, Protein stability, Cell growth/invasion	cABL	[249]
Ser236	DNA binding, Dimerization, Transcription	PKA	[262]
Ser282	Transcription	CK2	[248]
Ser305	DNA binding, Transcription, Coactivator binding, Interaction with other PTMs, Cell growth/invasion	PAK1, PKA	[263, 264]
Thr311	Transcription, Coactivator binding, Subcellular localization	p38 SAPK	[265]
Tyr537	Ligand binding, DNA binding, Dimerization, Transcription, Coactivator binding	c-SRC	[266]

Table 3: ERα phosphorylation sites and its function

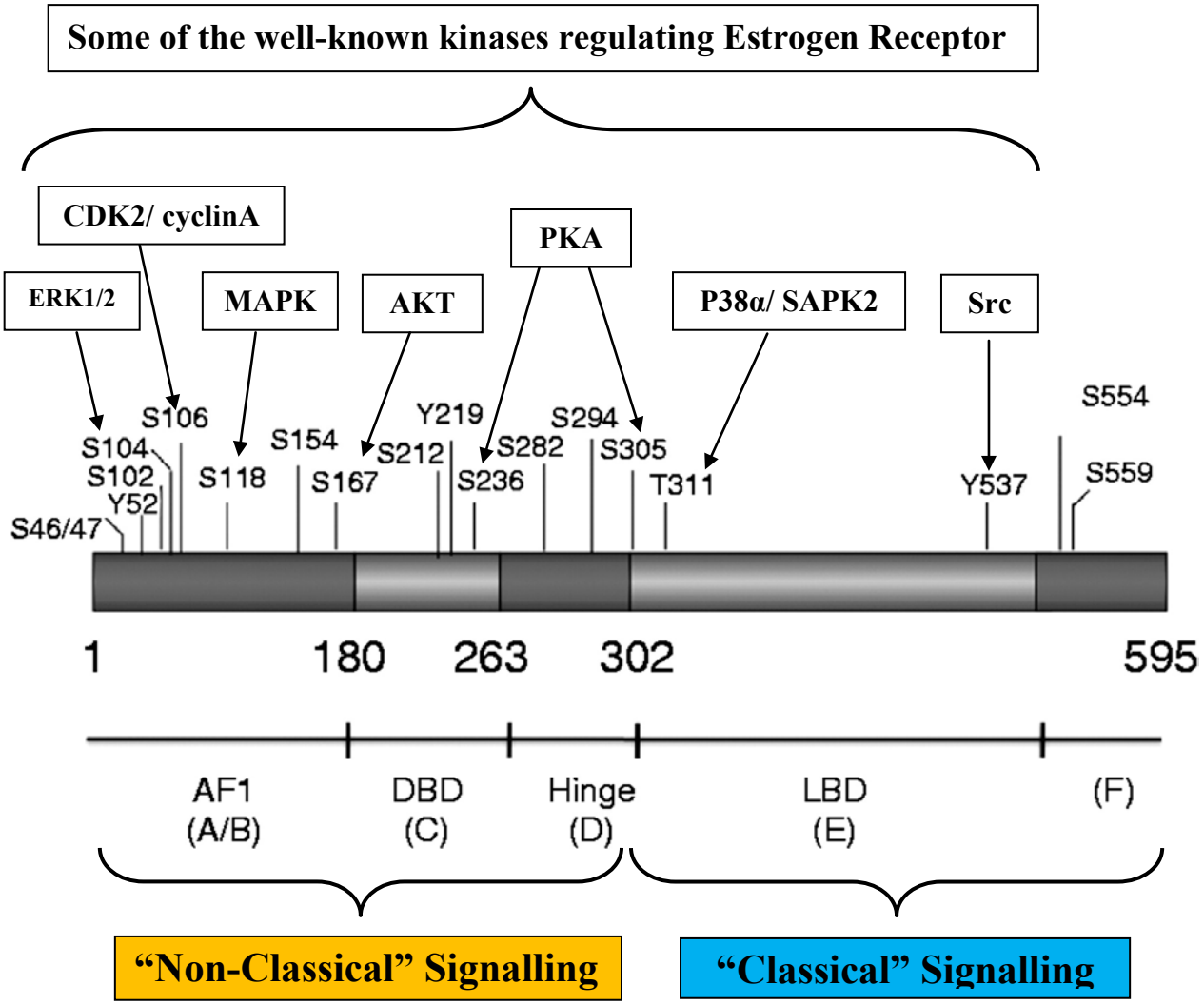


Figure 10: The known multiple phosphorylated sites in ERα have been identified and some of the well-known kinases that phosphorylates ERα. The different functional domains A/B, C and D promotes the non-classical signalling and domains E and F promotes the classical signalling of human ERα. Modified from [267, 268].

Resistance to endocrine therapy is a common problem and phosphorylation of ERα has a very crucial role in endocrine resistance [29]. Ser-118 is one the most important phosphorylation sites of ERα. It has been shown estrogen induces Ser-118 phosphorylation [269], kinases such as CDK7, MAPKs and GSK3 have all been shown to phosphorylate ERα on Ser-118 (Table 3). Other phosphorylation sites like Ser-167, Ser-236, Ser-305, Thr-311 and Tyr-537 have an important role in endocrine resistance [267]. Previous clinical studies suggest that the phosphorylation status is associated with good prognosis and response to endocrine therapy [270, 271]. Therefore phosphorylation of ERα could be one of the key mechanisms of resistance to endocrine therapy. The investigation of novel kinases involved in ERα phosphorylation may elucidate potential strategies for combating resistance to endocrine therapy.

1.10 siRNA Mediated Gene silencing or knockdown

siRNAs are double stranded RNAs, usually 20 to 25 nucleotides, involved in post-transcriptional gene silencing by binding to and promoting the degradation of mRNA at specific sequences [272, 273]. The discovery of small interfering RNAs (siRNAs) [274] has revolutionized our understanding of gene regulation. siRNA therapy is one the most powerful tools used in biological research over the last decade and has gained much attention for their ability to suppress gene expression by post-transcriptionally silencing a gene through mRNA degradation [275, 276]. siRNAs are able to suppress gene expression, without producing a significant cytotoxic response [277].

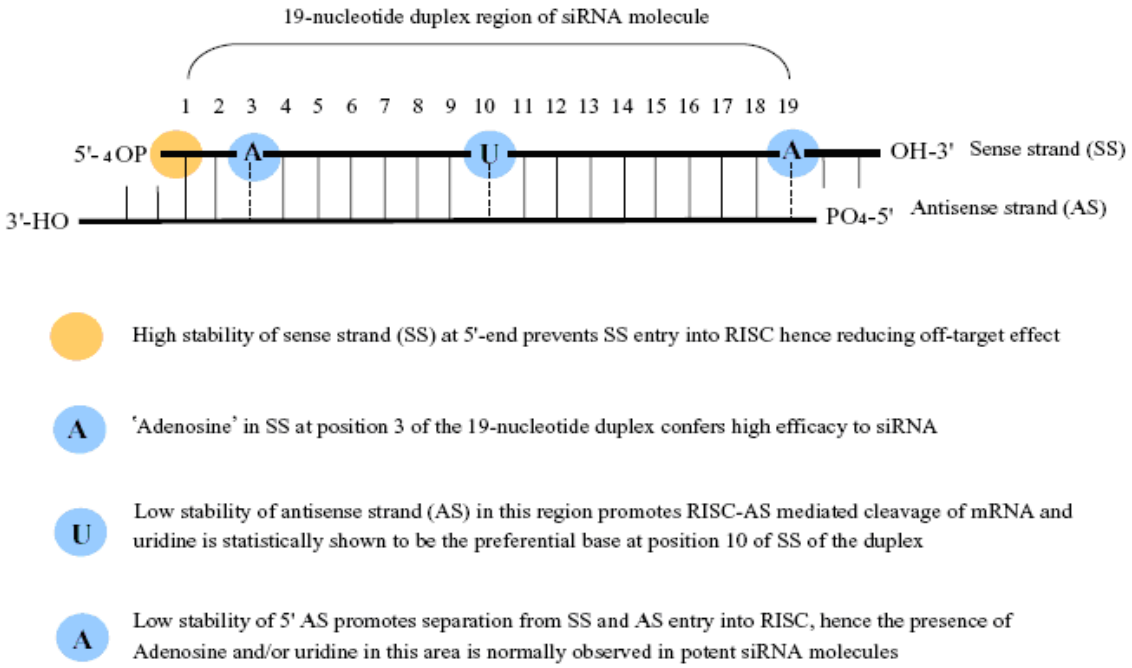


Figure 11: Structure of a siRNA. Positions of each nucleotide in the 19nt duplexed region of the sense strand of an siRNA; a model which shows to design an effective siRNA molecule. Adapted from [278].

1.10.1 Mechanism of siRNA (RNA interference)

In mammalian cells the RNase III enzyme Dicer recognises the siRNA duplex via the two nucleotide overhang and is incorporated into the RNA inducing silencing complexes (RISCs). Further, the ATP-dependent helicase unwinds the duplex and one of the strands independently recognizes mRNAs mediates siRNA mediated gene knockdown [278, 279]. The degree of complementarity between the guiding strand and the target mRNA determines whether mRNA silencing is achieved via the site-specific cleavage of the mRNA [280] or through a miRNA like mechanism by blocking the translation [281].

1.10.2 Off-target effects of siRNA

Initially it was thought siRNAs have laser-like specificity, but later expression profiling by Jackson *et al*. revealed the off-target effects of siRNAs [282]. It has been shown that a single siRNA duplex can target more than one mRNA transcript because of sequence homologies [283]. So therefore to minimise off-target effects and to maximize gene silencing, a careful selection of sequences is needed. Off-target activity of siRNAs can lead to unexpected phenotypes and complicate the understanding of the gene expression. The off-target effects of siRNA delivery fall under three categories; (a) siRNA mediated regulation of unintentional transcripts through partial sequence complementarity to their 3' UTRs (microRNA-like off-target effects) [284] (b) an inflammatory reaction through activation of Toll-like receptors triggered by siRNAs or their delivery vehicles [285] and (c) widespread effects on microRNA processing and function through saturation of the endogenous RNAi machinery by exogenous siRNAs [286].

1.10.3 RNAi screening for the discovery of novel modulators

Over recent years, the development of RNAi libraries (siRNA library) has changed the direction of drug target discovery and validation [287, 288]. RNAi technology has already influenced the strategies for drug development of many diseases including cancer [289]. Using RNAi libraries in various formats has led to the identification of novel regulators at genome wide level that alter the disease phenotype and also offers a better understanding of the mechanisms that trigger disease pathogenesis [290-293]. Earlier studies involving 'kinome screens' have

revealed critical kinases involved in the key pathogenesis of different cancers [294-298].

1.10.4 RNAi as the most potential therapeutic target

Initially it was thought that non-coding RNAs played no role in gene expression and were considered to be 'junk' molecules in the genome. However, the past 20 years has brought many discoveries about non-coding RNAs which have changed this previously held view. The discovery of RNA interference (RNAi) has revolutionized the understanding of gene regulation [299]. siRNAs (exogenous) and miRNAs (endogenous) are the central players in RNAi; and are considered to be both a valuable tool in understanding biological mechanisms, and also potential therapeutic targets.

1.11 Aims

The aims of this research are:

1. The first aim of this project was to identify specific miRNAs that are transcribed and then regulated in breast cancer in an estrogen dependent manner and to determine the contribution of post-transcriptional miRNA regulation to oncogenesis in breast cancer, in response to ERα's transcriptional activity. Also, to establish the mechanisms of how miRNAs are regulated in breast cancer and identify any miRNA-binding proteins involved.

2. The aim of the second part of the project was to screen the entire 'kinome' family and identify novel kinases regulating estrogen receptor using RNAi technology, which may provide valuable information to design novel therapeutic strategies against ER positive patients.

Chapter 2: Methods and Materials

2.0 Methods and Materials

2.1 Cell Culture and passaging cells

MCF-7 and MELN cells were maintained in DMEM (Gibco, Invitrogen) supplemented with 10% FCS, 1% penicillin/streptomycin and 1% glutamine. MCF-7-Tet-Off (MCF-7-TO) cells (BD Biosciences, UK) were maintained in DMEM supplemented with 10% FCS, 100µg/ml G418 and 1µg/ml of doxytetracycline (Tet). MCF7-TO cells (BD Biosciences, UK) in DMEM supplemented with 10% FCS, containing 100 mg/ml G418 and 1 mg/ml of the more stable tetracycline analogue doxytetracycline (Tet). MCF7-TO-PLZF-ERα cells were maintained as previously described [300].

Cells were cultured in 150cm^2 flasks or 100-mm dishes or 6-well plates unless otherwise specified and maintained at 37°C in a humidified 5% CO2 incubator. Cells were routinely passaged when a confluency of ~90% was reached, depending on the growth curve of each cell line. To passage cells, medium was aspirated, cells were washed once with PBS solution and then trypsinised with EDTA-trypsin at 37°C for 3 to 10 minutes to allow them to detach. FCS was added to inactivate the trypsin (1:1 ratio) and cell clumps were disrupted through gentle pipetting. The resulting liquid suspension of deaggregated cells was pipetted out of flask, and transferred to 15 mL sterile centrifuge tube. The cell suspension was centrifuged for 5 min at 1300 rpm. After centrifugation, the supernatant was aspirated and the cell pellet resuspended in an appropriate volume of medium. The resulting suspension was split to the desired dilution into new flasks and fresh media was added.

2.2 Cell treatments and RNA isolation

For E2 treatment, 1.5 x 10^6 of MCF-7 cells were deprived of hormones through growth in DMEM lacking phenol red (Gibco, Invitrogen), supplemented with 10% dextran-coated charcoal-treated FCS (DSS) for 72 h then stimulated with E2 (10 nM) for 0, 3, 6 or 12 h, at which points total RNA was isolated using Trizol (Invitrogen, UK). For the MCF-7-TO and the MCF-7-TO-PLZF-ERα lines, 1.5 x 10^6 cells were seeded in 10 cm plates. After 24 h, the cells were washed with PBS and DMEM lacking phenol red, supplemented with 10% DSS and G418. Cells were cultured for a further 72 h, at which time E2 (10 nM) and Tet (1 μg/ml) were added as indicated. These cells were maintained in E2 for 24 h before Trizol RNA extraction [1].

For separation of small (< 200 nt) and large (> 200 nt) RNA fraction the miRNeasy mini kit was used for obtaining the larger fraction, followed by the RNeasy MinElute Cleanup kit to isolate the smaller fraction, according to the manufacturer's instructions (Qiagen, Crawley, UK). Separation of RNA between the large and small fractions was confirmed by gel electrophoresis. After extraction the quality and levels of RNA were determined using an Agilent Bioanalyzer (Agilent Technologies, Edinburgh, UK) and a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Horsham, UK) [1].

Cells were lysed directly in the well by adding 1ml of Trizol reagent and the lysates were collected in Eppendorf tubes and mixed by pipetting. 200μl of chloroform per 1ml of Trizol reagent were added to the lysates. The samples were vortexed vigorously for 15 seconds, incubated at RT for 2 to 3 minutes, and

then centrifuged at 12,000 x *g* for 15 minutes at 2 to 8°C. Following centrifugation, each mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. Each aqueous phase was transferred carefully without disturbing the interphase into fresh Eppendorf tubes. The total RNA was precipitated from the aqueous phases by mixing with 500µl of isopropyl alcohol and incubating for 10 minutes at RT. The mixtures were then incubated overnight at -80 °C to allow small RNA precipitation. After incubation, the RNA precipitates were centrifuged at 12,000 x *g* for 10 minutes at 2 to 4°C to form a pellet on the side and bottom of the tube. The supernatants were removed, and the RNA pellets were washed by adding 1ml of 75% ethanol and vortexing. The samples were centrifuged at 7,500 x *g* for 5 minutes at 2 to 8°C. The supernatants were removed and any residues of ethanol were air-dried. Finally the RNA pellets were resuspended in an appropriate volume of RNAse-free water. Subsequently, RNA concentration was measured at 260nm and 280nm wavelengths, using a NanoDrop ND-100-Spectrophotometer (NanoDrop Technologies).

2.3 miRNA microarray

The microarray was done in collaboration with Genomics Laboratory, Hammersmith Hospital, Imperial College London. Isolated RNA was labelled using the Agilent labelling kit following the manufacturer's instruction (Agilent Technologies, USA). The Agilent human (V1) miRNA Microarray platform, containing probes for 470 human and 64 viral miRNAs from the Sanger database

v9.1, was used to perform miRNA expression profiling. After hybridization, microarrays were scanned with Agilent scanner (G2565BA) using Agilent Feature Extraction software. Raw data were imported and analyzed by the Resolver gene expression data analysis system version 4.0 (Rosetta Biosoftware, Seattle, WA). A two-tailed Student *t*-test was performed with a *p*-value cutoff of 0.01 was used to identify significantly changed miRNAs. Three biological replicates were used in each experiment.

2.4 Transfection and luciferase reporter assays

MELN cells (5 x 10^5) were plated in 24 well plates in medium containing DMEM/10 % DSS for 24 h, transfected with either the pre-miR miRNA precursor or an anti-miR miRNA inhibitor (Applied Biosystems) for 48 h using HiPerFect Transfection Reagent (Qiagen) and treated with E2 (10 nM) or vehicle (ethanol) for 24 h before luciferase measurement. For the 3'UTR reporter assay, 200 ng pMIR-REPORT *firefly* luciferase vector, including the various fragment of *ERα* 3'UTR, 100 ng of *Renilla* luciferase vector (pRL-TK) and the pre-miRNAs or negative control oligonucleotide, were transfected using Lipofectamine 2000 (Invitrogen). Forty eight hours after transfection, cells were harvested and luciferase activity measured using the Dual-Glo luciferase assay system (Promega).

MCF7 cells were plated in 24-well tissue culture plates at a density of 5 x 10^4 cells/well in 0.5mL of medium without antibiotics. Cells were allowed to adhere and grown at 37 °C with 5% CO_2 for 24 hours and then transfected accordingly. After forty eight hours of transfection, cells were lysed with 50µl/well of the Cell

Culture Lysis Buffer (5x) (Promega) fivefold diluted in dH_2O and placed on agitator at constant speed for 30 minutes. Luciferase assay was performed by using the Dual-Glo Luciferase assy system (Promega). The lysates were transferred in a Opti-plate 96-well and mixed with 50µl/well Dual-Glo™ Luciferase Reagent. After 10 minutes, the 96-well plate was sealed and the *firefly* luciferase activity was measured by using a luminometer. *Renilla* luciferase luminescence was measured by adding 50µl/well of Dual-Glo™ Stop & Glo® Reagent, prepared according to the manufacturer's instructions, and after 10 minutes, the reading of *Renilla* luminescence was taken by a luminometer. The ratio of luminescence from the experimental reporter to luminescence from the control reporter was then calculated.

2.5 Protein Analysis

2.5.1 Preparation of cell lysates

Cells were plated in 100-mm tissue culture dishes or in 6-well tissue culture plate for the pre-miRNAs over-expression, allowed to adhere and grown for 48 hours. The dishes were placed on ice and cells were washed once with cold PBS. Cells were scraped in cold PBS and centrifuged for 5 minutes at 1300 rpm. The supernatant was removed and the cell pellets was lysed in NP-40 lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10 % (v/v) glycerol, 1 % NP40, 5mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA) + protease inhibitors cocktail solution (Roche). Eppendorf tubes containing cell lysates were placed on a rotator for 15 minutes at 4°C. A microcentrifugation of the lysates at maximum speed (13,000 rpm) for 15 minutes at 4°C allowed the separation of protein from

insoluble elements. The supernatants containing proteins were transferred in new Eppendorf tubes and subsequently subjected to protein quantification.

2.5.2 Protein quantification

The protein concentration in the supernatants was determined using the Bradford Reagent (BioRad) diluted tenfold in dH_2O. In reading cuvettes, a standard solution and a solution used as "blank" were prepared by adding 1µl of 1mg/ml BSA (Invitrogen) and 1µl of the NP-40 working lysis buffer, respectively, in 1ml of diluted Bradford Reagent. Protein samples were diluted 1:10 and 1µl was added in 1ml of diluted Bradford Reagent. Absorbance readings were measured at 595nm using a UV/Visible spectrophotometer (Beckman DU® 530 Life Science). Upon collection of the data, the concentration of the unknown samples was determined based on standard absorbance value. The protein samples were then prepared with the SDS Loading Buffer (2x) and boiled at 95°C for 5 minutes.

2.5.3 Western blots

Both acrylamide (6-20%) resolving and 4% stacking gels were prepared manually as required. The rainbow marker (Fermentas, life science) and protein samples (10µl) were loaded, electrophoresis was carried out for 2 to 3 hours at 80V. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 1X SDS running buffer.

Proteins were transfered to Hybond C super nitrocellulose membrane (GE Healthcare) at 100V for one hour in 1X transfer buffer using a Mini-PROTEAN®

Tetra Cell (BioRad). Following brief incubation with Ponceau S solution (Fluka) to check sufficient transfer, membranes were washed in 0.1% TBS-Tween (TBST), and incubated in 5% BSA in 0.1% TBST on a rocker shaker at 4°C for 1 hour. Membranes were probed using antibodies diluted in 5% BSA in 0.1% TBST overnight. We probed with AIB1, ERα, β-actin, ALY (mouse monoclonal antibodies) purchased from Abcam (Cambridge, UK). TBST was used to wash the membrane for three times, 15 minutes each. An IgG/HRP secondary antibody (DAKO, UK) diluted in blocking solution was then added, and the membrane was incubated at room temperature for 1 hour. The membrane was washed 3 times with TBST and Enhanced Chemiluminescence (ECL) detection system (GE Healthcare) was used for visualization. The emitted fluorescence was detected using Hyperfilm ECL (GE Healthcare) on SRX-101A x-ray developer. Band intensities were measured by the image analysis program Image J software (NIH, Bethesda, MD).

2.6 Northern Blotting

RNA was extracted using Trizol, total RNA (30µg per lane) was loaded on 15% denaturing polyacrylamide gel and electophoresed at 300V until the bromophenol blue approached the bottom. After electrophoresis the RNA was transferred from the gel to Hybond-N+ (GE Healthcare) membrane using a wet transfer apparatus. DNA oligonucleotide probe complementary to miR-18a, mir-21, mir-34a and U6 were 5' -end labeled with [γ-^{32}P] ATP, and hybridization was performed using Miracle hybridisation buffer according to the manufacturer's

instructions (Thermo Scientific). After hybridization, membranes were washed three times with 2× SSC and 0.05% SDS, twice with 0.1× SSC and 0.1% SDS, and exposed from overnight up to 1 week depending on the expression of the miRNA.

2.7 Reverse Transcription and Real Time PCR

2.7.1 cDNA synthesis for Taqman probe

The reverse transcription of mature miRNAs was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). 10ng of total RNA were mixed with 7µl of master mix and 3µl of microRNA-specific RT TaqMan Probes (Applied Biosystems) in a 48-well PCR plate (Thermo Scientific, Abgene®). The samples were incubated in the 7900Ht Thermal Cycler (Applied Biosystems) at 16°C for 30 minutes to allow primers to anneal, followed by 30 minutes at 42°C for the elongation step, and 5 minutes at 85°C to inactivate the reverse transcriptase. After RT cycles, the cDNAs samples were placed in ice and then prepared for quantitative real-time PCR.

2.7.2 Quantitative real-time PCR by Taqman probe

In order to amplify mature miRNAs, for a single reaction, 1ng of relative cDNA template was distributed in a Fast Optical 96-well reaction plate (Applied Biosystems), followed by the appropriate volume of a master mix. The master mix was prepared by combining 10µl of 2x TaqMan® Universal PCR Master Mix, No Amperase® UNG (Applied Biosystems) with 1µl of the relative 20x Real Time TaqMan probe (Applied Biosystems), by adding ddH2O to a final volume of

15.57µl per single reaction. Each reaction was done in triplicate. The plate was then sealed using the Optical Adhesive Cover Starter Kit (Applied Biosystems) and centrifuged (2,000 x g) for 30 seconds at 4°C. Quantitative real-time PCR (qPCR) was performed with an ABI Prism 7900HT sequence detection system (Applied Biosystems) with a thermal cycling program as follow: a first stage of 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Finally, data were analyzed using SDS 3.1 software (Applied Biosystems).

2.7.3 cDNA synthesis for Sybr green

The reverse transcription of Primary (pri) and Precursor (pre) transcripts was performed using the SuperScript III First-Strand cDNA synthesis system (Invitrogen). 1µg of purified DNAse treated RNA were mixed with reverse primers in a 48-well PCR plate (Thermo Schientific, Abgene®). The samples were incubated in the 7900HT Thermal Cycler (Applied Biosystems) at 65°C for 10 minutes to open up the secondary structures and also for primers to anneal. Then 6µl of master mix was added to make a final volume of 10µl. Cycling conditions for the reaction were 55°C for 30 minutes and 5 minutes at 85°C to inactivate the reverse transcriptase. After RT cycle, the cDNAs samples were placed in ice and then prepared for quantitative real-time PCR.

2.7.4 Quantitative real-time PCR by Sybr green

In order to amplify the primary and precursor transcripts, 10ng of cDNA template was distributed in a Fast Optical 96-well reaction plate (Applied Biosystems), followed by the appropriate volume of a master mix. The master mix was prepared by combining 10µl of 2x Power SYBR green PCR master mix (Applied Biosystems) and 10µl of cDNA/ primers, add ddH2O to a final volume of 20µl per single reaction. Each reaction was done in triplicate. The plate was then sealed using the Optical Adhesive Cover Starter Kit (Applied Biosystems) and centrifuged (2,000 x g) for 30 seconds at 4°C. Quantitative real-time PCR (qPCR) was performed with an ABI Prism 7900HT sequence detection system (Applied Biosystems) with a thermal cycling program as follow: a first stage of 10 minutes at 95°C followed by 40 cycles of 20 seconds at 95°C 20 seconds, 20 seconds at 60°C and 20 seconds at 72°C. Data were analyzed using SDS 3.1 software (Applied biosystems).

2.8 ChIP (Chromatin Immunoprecipitation)

MCF-7 cells were first cross-linked using 37% Formaldehyde. The cells were then resuspended in solution 1 (0.25% Triton X-100, 10 mM EDTA, EGTA 0.5 mM, 10 mM Tris-HCl pH 8), and fresh protease inhibitor cocktail (Roche) was added. The cells were rotated at 4^{O}C 10 min, spun down and then solution 2 (0.2 M NaCl, 10 mM EDTA, 0.5 mM EGTA, 10 mM Tris-HCl pH 8) was added. The cells were rotated at 4^{O}C 10 min, spun down again and resuspended in

sonication buffer (SDS – 1%, EDTA – 10mM, Tris HCl pH 8 – 50 mM). The samples were sonicated (at a frequency predetermined to produce appropriately sized DNA fragments) and resuspended in ChIP buffer (0.01% SDS, 1.1% Triton-X100, 1.2mM EDTA, 16.7mM Tris-HCl, pH8.1, 167mM NaCl). Each aliquot cross-linked chromatin (20 µg) was precleared with 25 µl of Protein A-Sepharose beads (Amersham Biosciences). Aliquots were incubated overn

ight with 2 µg of c-Myc (sc-764) and ERα (sc-543) antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) or without (mock controls) in a total volume of 1 ml and immunoprecipitated. Reverse histone-DNA crosslinking was done by adding 5M NaCl (5µl) to the eluate and input samples and incubated at 65^0C overnight. RNase (Qiagen, 100mg/ml) was added to samples (final concentration – 50µg/ul) to degrade RNA, incubated at 37 ^{0}C for 30 minutes and the DNA was purified with phenol chloroform extraction. Triplicate samples of 5 µl of immunoprecipitated genomic DNA were amplified by real time PCR. Values are expressed as fold of enrichment with respect to input DNA. Primer sequences used in this assay are listed in Table 2.

2.9 Breast cancer specimens

Snap frozen breast cancers derived from post-menopausal women with strongly ERα positive (immunohistochemistry 3+) or negative (control group) primary invasive ductal carcinomas were included, as described [301]. Total RNA was extracted from ~500 µg of frozen tissue using Trizol (Invitrogen). For quantification of pre-miR-18a, U6 and HNRPA1, cDNA was synthesized from 1 µg

of purified DNAse treated RNA by the SuperScript III First-Strand cDNA synthesis system (Invitrogen) and qRT-PCR was performed on a 7900HT Thermocycler using the Power SYBR green PCR master mix (both from Applied Biosystems). Appropriate ethical approval was obtained.

2.10 EMSA (Eletrophoeretic Mobility Shift Assay)

EMSA were performed as described previously [302]. Briefly, binding reactions were carried out in (20 mM HEPES, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 µg/µl BSA and 10% glycerol), final reaction volume of 20 µl for 30 min at 37°C. γ-^{32}P labeled single-stranded probes (Table 7) were added to a final concentration of 5nM together with recombinant Aly/Ref. Reactions were subjected to electrophoresis on 12% polyacrylamide gels containing 5% glycerol in TBE (0.5X) and run for 2 hours at 300V until the bromophenol blue approached the bottom of gel. After electrophoresis, gels were dried and exposed to Hyperfilm ECL (GE Healthcare).

2.11 Co-Immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed to detect protein-protein interactions. MCF-7 cells were starved in DMEM lacking phenol red (Gibco, Invitrogen), supplemented with 10% dextran-coated charcoal-treated FCS (DSS) for 72 h and then stimulated with E2 (10 nM) for 3h with ethanol used as an (E2-) control treatment ('vehicle'). Cells were treated as indicated in the text and lysed by sonication in Marais' lysis buffer (25 mM HEPES [pH 7.8], 400 mM KCl, 5 mM EDTA, 0.4% NP40, 10% glycerol freshly supplemented with 1 mM DTT,

protease, and phosphatase inhibitors). The extracts were diluted fourfold to bring KCl concentration to 100 mM and NP40 to 0.1%, supplemented with 0.5% BSA (Roche, UK) and 10 mM $MgCl_2$. The extracts were incubated on ice for 15 minutes and spun at the max speed (13,000 rpm) at 4°C; the supernatant (lysates) was removed and transferred to a new eppendorf. The lysates were further with incubated with protein-A sepharose beads and 5µg of flag-Dicer ab (M2 clone, sigma-aldrich, USA) for immunoprecipitation, overnight at 4°C. Beads were quickly washed three times at RT with wash buffer (100 mM KCl, 0,05% NP40) and subjected to centrifugation (13,000 rpm) to remove the supernatant. The beads were then boiled at 95°C for 5 minutes with Laemmli buffer before separation on a SDS-PAGE gel.

2.12 Mass Spectrometry Analysis by HPLC MS/MS

The Mass Spectrometry was done in collaboration with Dr. Robin Wait, Charing Cross Hospital, Imperial College London. This protocol was kindly provided by Dr. Robin Wait.

Protein identification and relative quantitation were performed by liquid chromatography mass spectrometry using the isobaric tags for relative and absolute quantitation (iTRAQ) method. This is an amino tagging strategy which employs up to eight isobaric but isotopically distinguishable derivatization reagents, which are coupled to free amino groups of sample peptides. We performed a series of duplex comparisons [Estrogen stimulation (E2) vs Vehicle (E2-); pre-miR-18a over expression vs pre-miR-34a over expression (control

bait)]. MCF7 cells (2 X 10^7) were lysed in 250 μl 0.5 M triethylammonium bicarbonate /0.05% SDS at 4° C, centrifuged and protein quantified by Bradford assay. After cysteine reduction and alkylation 100 μg aliquots of protein were digested with trypsin overnight at 37° C. Each 50-100 μg sample will then be derivatised with 1 unit of label (iTRAQ-4plex; Applied Biosystems, Warrington UK) for 1 h at room temperature.

The samples resulting from each binary comparison experiment were pooled, and fractionated prior to LC MS by off line strong cation exchange chromatography. Eight fractions from 50mM to 500mM salt were collected, resuspended in 0.1% formic acid and loaded onto a 15 cm x 75μ C18 column (LC Packings pepmap) installed in a Dionex Ultimate 3000 HPLC system. Peptides were separated with acetonitrile 0.1% formic acid gradient into a Q-Star XL mass spectrometer (Applied Biosystems) fitted with a nanoflow ESI source (Protana/Applied Biosystems), and tandem mass spectra obtained by automated data dependant acquisition. Protein identification and quantitation were performed using both proQuant software (Applied Biosystems) and our local Mascot server using 4-plex iTRAQ reagents as fixed modifiers [303] [304]

2.12.1 Identification of gel separated proteins

Proteins bands were excised from the gels and digested with trypsin as described previously [304] and analysed using a Q-Tof spectrometer (Micromass, Manchester, UK) interfaced to a Micromass CapLC capillary chromatograph fitted with a 300 μm x 5 mm Pepmap C18 column (LC Packings, Amsterdam, NL). Data

dependent acquisitions were performed on precursor masses with charge states of 2, 3 or 4, over the *m/z* range 400-1300 using argon collisional activation, and proteins were identified by correlation of uninterpreted spectra to entries in SwissProt/TrEMBL, using Mascot version 2.2 (Matrix Science, London).

2.13 Transfection of Plasmid DNA

Cells were seeded in 10cm dishes to confluency of approximately 90% with DMEM medium without any antibiotics. Lipofectamine® 2000 (Invitrogen) was used as the transfection reagent. The ratio 3:1 (ul of Lipofectamine® 2000: µg of DNA) was used, as recommended by the manufacturer. To begin, 975 µl of optimem was pipetted into a sterile eppendorf tube. 18µl of Lipofectamine® 2000 were then carefully added and the mixture was shaken slightly and left at RT for 5 minutes. To this 6µg of plasmid DNA was added, gently mixed and left at room temperature for 20 minutes. The mixture was then pippetted dropwise into the 10cm dish while gently being shaken for uniform distribution of the transfection complex. Overexpression of desired proteins was confirmed 48h after transfection, by real-time and western blot analysis

2.14 RNA Interference (RNAi) – Kinome Screening

siRNA library (Human Kinase siRNA Set V3.0, Qiagen) containing 691 kinases was used to specifically repress the expression of entire kinome. In this study, all siRNAs used were pooled siRNAs which had 2 siRNAs targeting two independent sites on the same gene. HiPerFect (Qiagen) was used as the transfection reagent. MCF7 cells 40,000 per well of a 24-well plate, were plated in DMEM

lacking phenol red (Gibco, Invitrogen), supplemented with 10% dextran-coated charcoal-treated FCS (DSS) 24h prior to transfection. For each well, 20nM of siRNA was added to 85µl of neat DMEM medium to clean eppendorf and then 3µl of HiPerFect was added to each tube. The mix was incubated at room temperature for 10min. After incubation, the mix was added drop-wise to the wells and the plates were shaken for uniform distribution of the transfection complex. Cells were also transfected with appropriate controls provided together with the siRNA libarary. After 48h of transfection, the cells were treated with Estradiol (E2) and Vehicle (Ethanol) respectively. Further after 24h of E2 stimulation the cells (in total 72h of transfection) were subjected to total RNA extraction.

2.15 Total RNA Extraction – for Kinome Screening

After 60h of transfection with siRNAs, total RNA was isolated from MCF7 cells using the RNeasy Kit (Qiagen, Crawley, UK). The protocol was performed in line with the manufacturer's instructions using QIAcube (Qiagen, USA). Cells were resuspended in 350µl of RLT buffer (containing 10% ßmercaptoethanol), homogenised with 1000 µl pipette and the lysates were transferred to the QIAshredder spin column and 350 µl of 70% ethanol was added, mixed by pipetting. The total mixture was added to the provided column placed in a 2ml collection tube and spun at 13,000rpm for 30s, and the flow through was discarded. 700µl of buffer RW1 was added to the column and was spun at 13,000rpm for 30s, the flow through was again discarded. Next, 500 µl of RPE buffer was added to the column and microcentrifuged at 13,000 rpm for 30s, the

flow through was discarded and the column, spun for an additional 2 minutes at 13,000 rpm to extract any waste from the column. The column was then transferred to a clean eppendorf tube. 30µl of RNase-free water was carefully added to the centre of the column and was spun for 1 minute at 13,000 rpm to elute the extracted RNA. The purity and concentration of the RNA was then determined using Nanodrop, which measures the spectrometric absorption at 260nm and 280nm. RNA samples were then stored at -80ºC.

2.15.1 Reverse Transcription and Real Time PCR – Kinome screening

The Reverse transcription was performed using high capacity cDNA reverse transcription kit (Applied Biosystems). 1µg of total RNA were mixed with MultiScribe™ Reverse Transcriptase, dNTPs and random primer mix in a 48-well PCR plate (Thermo Schientific, Abgene®). The samples were incubated in the Veriti® 96-Well thermal Cycler (Applied Biosystems) at 25°C for 10 minutes to allow primers to anneal, followed by 120min at 37°C for the elongation step, and 5 minutes at 85°C to inactivate the reverse transcriptase. After RT cycles, the cDNAs samples were placed in ice and then prepared for quantitative real-time PCR.

2.15.2 Quantitative real-time PCR by taqman probe - Kinome screening

In order to amplify single gene expression, 5ng of cDNA template was distributed in a Fast Optical 96-well reaction plate (Applied Biosystems), followed by the appropriate volume of a master mix. The master mix was prepared by combining 10µl of 2x TaqMan® Universal PCR Master Mix (Applied Biosystems) with 1µl of

the relative 20x Real Time TaqMan probe (primers for TFF1, PGR, GREB1, LMTK3, and GAPDH, purchased from Applied Biosystems), and finally by adding appropriate volume of ddH2O to make final volume of 20µl per single reaction. Each reaction was done in triplicate. The plate was then sealed using the Optical Adhesive Cover Starter Kit (Applied Biosystems) and centrifuged (2,000 x g) for 30 seconds at 4°C. Quantitative real-time PCR (qPCR) was performed with an ABI Prism 7900HT sequence detection system (Applied Biosystems) with a thermal cycling program as follow: a first stage of 10 minutes at 95°C followed by 40 cycles of 20 seconds at 95°C, 20 seconds at 60°C and 20 seconds at 72°C. Finally, data were analyzed using SDS 3.1 software (Applied biosystems).

2.16 Materials

2.16.1 Buffers and Solutions

Table 4: Lists of buffer and reagents

Reagent	Recipe	Storage
PBS (Phosphate buffered Saline)	5 PBS tablets (Sigma) dissolved in 500ml of ddH_20	4°C
EDTA-Trypsin	2.5g/l Trypsin in 0.02% EDTA (Sigma-Aldrich)	4°C
1M DTT (Dithiothreitol)	1.54g of DTT in total of 10ml ddH_20 (double distilled MilliQ water)	100µl aliquots at -20°C
75% Ethanol	75ml Ethanol added to 25ml of ddH_20	Room temperature
1M Tris-HCl	60.5g Tris in total of 500ml ddH20 and adjusted to desired pH with pure HCl	Room temperature
Laemmli's buffer	50mM Tris-HCl pH 6.8, 15% glycerol, 0.1% (w/v) bromophenol blue, 4% SDS (BioRad)	Room temperature

SDS (Sodium Dodecyl Sulphate) **loading buffer (2x)**	125mM Tris-Cl pH 6.8, 20% glycerol, 4% SDS, 100mM DTT, 0.04% (w/v) bromophenol blue	500µl aliquots at 4°C
10x SDS PAGE Running buffer	10g SDS, 30.3g Tris, 144.1g glycine dissolved in 1l of ddH_20	Room temperature
10x Transfer buffer	30.3g Tris, 144,1g glycine dissolved in 1l of ddH_20	Room temperature
TBS 10x (Tris-Buffered Saline)	24.23g Trizma HCl, 80.06 g NaCl dissolved in 1l of ddH_20 and adjusted pH to 7.6 with pure HCl	Room temperature
TBST buffer (TBS/ Tween® 20)	100ml of TBS 10x, 900ml ddH20, 1ml Tween® 20 (BDH)	Room temperature
Blocking solution	5% dried skimmed milk powder in TBS/ Tween	Prepared as required

2.16.2 Primers

Most of the Primers were synthesized from Eurofins and some of them by Sigma-Aldrich.

Table 5: Primer sequences

Gene	Forward primer	Reverse primer
GREB1	CAAAGAATAACCTGTTGGCCCTGC	GACATGCCTGCGCTCTCATACTTA
GAPDH	TGAAGGTCGGAGTCAACGGATTT	GCCATGGAATTTGCCATGGGTGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
HNRPA1	ATACTGTGAATGGCCACAA	AACCACTTCGACCTCTTTG
ERα	GAATCTGCCAAGGAGACTCGC	ACTGGTTGGTGGCTGGACAC
AIB1	CGTCCTCCATATAACCGAGC	TCATAGGTTCCATTCTGCCG
TIF2	GCCCGATTTCTCTTGGATTTG	TGGAGGGGTCAGAGGTATTT
SRC1	ATGGTGAGCAGAGGCATGACA	AAACGGTGATGCTCATGTTG
ER1	CGCACTAGTAAATGGCTCTAAGAATAAGC	CGCACGCGTAGTGCTATTTTGTCTACTGT
ER2	CGCACGCGTGATGCCTATTGTTGGATACT	CGCACGCGTAATTGTTTACAGGTGCTCGA
ERup	CGCACGCGTAGTGCTATTTTGTCTACTGT	CGCACTAGTCCACACGGTTCAGATAAT
ERfl	CGCACGCGTAGTGCTATTTTGTCTACTGT	CGCACGCGTAATTGTTTACAGGTGCTCGA
TFF1 promoter	CACCCCGTGAGCCACTGT	CTGCAGAAGTGATTCATAGTGAGAGAT
mir-17-92 promoter	AAAGGCAGGCTCGTCGTTG	CGGGATAAAGAGTTGTTTCTCCAA
pri-miR-17-92	CAGTAAAGGTAAGGAGAGCTCA	CATACAACCACTAAGCTAAAGAA
pre-miR-18a	TAAGGTGCATCTAGTGCAGATAG	GAAGGAGCACTTAGGGCAGT
pri-miR-34a	CAACCAGCTAAGACACTGCCAA	CCTCCTGCATCCTTTCTTTCCT
pre-miR-34a	TGGCAGTGTCTTAGCTGGTTG	GGCAGTATACTTGCTGATTGCTT
pri-miR-342	GCCATTGCATCCTTCTCT	TCAATCACAGATAGCACCC

RBM34	GCCTGTGCCTAAACAAACC	CTTGCGAAAGTGGTCTTTCA
TNRC6B	GTTTCAAAGAAGGGTGCTG	ACAAACATCCTCATGCTTCA
METTL3	TCGTTAGAAGCTGAAGGAAG	ACAGGTTCAGTTCAATCACA
CTTN	TCCCAGAAAGACTACTCCA	TGTCGATACCGTATTTGCC
SF3B3	GCCCGACTTACCAATTCA	TGTCTGCTTCCTCATAATCC
ANKRD17	AGTCCCTCAGGTATTGTCA	CATCGTACCTCCTGAAATGT
DAZAP1	GAAGGAAGGATGGCAGAAA	GGAAGGATGGCAGAAAGG
ALY/REF	CGTGGAGACAGGTGGGAAAC	GCACAGCCGCCTTCTTCAG
DICER	GTACGACTACCACAAGTACTTC	ATAGTACACCTGCCAGACTGT

Table 6: Northern Blot Primer sequences

miR	Northern Blot primers
miR-18a	TATCTGCACTAGATGCACCTT
miR-21	TCAACATCAGTCTGATAAGCT
miR-34a	ACAACCAGCTAAGACACTGCC
U6	GAATTTGCGTGTCATCCTTGCGCAGGGGCCATGCTAA

Table 7: Nucleotides Used in EMSA assay

MiR	Nucleotides Used in EMSA
miR-18a ss	UAAGGUGCAUCAAGUGCAGAUAG
miR-18a*	ACUGCCCUAAGUGCUCCUUCUGG
miR-18a TL (Terminal loop)	UGAAGUAGAUUAGCAUCU
Pre-18a	CCAGAAGGAGCACTTAGGGCAGTAGATGCTAATCTACTTCACTAT

Table 8: siRNA sequence of the kinases whose knockdown down-regulated *TFF1* expression

Gene	Gene Description	siRNA sequence
ABL1	v-abl Abelson murine leukemia viral oncogene homolog 1	ACGCACGGACATCACCATGAA
ACVR1	activin A receptor, type I	CTGGTCTGTCTTTGGATAATA
ACVR2B	activin A receptor, type IIB	TACGGTCATGTGGACATCCAT
AK1	adenylate kinase 1	CCGGGATGCCATGGTGGCCAA
AKAP1	A kinase (PRKA) anchor protein 1	AGCGCTGAACTTGATTGGGAA
AKAP13	A kinase (PRKA) anchor protein 13	CACGGTCATTATGAGAAACAA
AKT2	v-akt murine thymoma viral oncogene homolog 2	CAAGCGTGGTGAATACATCAA
AKT3	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	ACCAGAGGTGTTAGAAGATAA
ATM	ataxia telangiectasia mutated (includes complementation groups A, C and D)	AAACTACTGACTCGTGTATTA
ATR	ataxia telangiectasia and Rad3 related	GACCGGATACTTACAGATGTA
BCR	breakpoint cluster region	CAGCATTCCGCTGACCATCAA
CAMK1G	calcium/calmodulin-dependent protein kinase IG	CAGGTCTTGTCGGCAGTGAAA
CAMK2A	calcium/calmodulin-dependent protein kinase (CaM kinase) II alpha	CAGGAACTTCTCCGGAGGGAA
CDC2L1	cell division cycle 2-like 1 (PITSLRE proteins)	CAGGATCGAGGAGGGCACCTA
CDKN2A	cell division cycle 2-like 1 (PITSLRE proteins)	CAGGATCGAGGAGGGCACCTA
CIT	citron (rho-interacting, serine/threonine kinase 21)	CAGGATATACCGTAACACGAA
CKMT1B	creatine kinase, mitochondrial 1B	ACGGATCTAGATGCCAGTAAA
DAPK1	death-associated protein kinase 1	AAGCATGTAATGTTAATGTTA
EPHB1	EPH receptor B1	ATGGCCCTGGATTATCTACTA
GALK1	galactokinase 1	CCGCCTCATGGTGGAGAGCCA
GRK6	G protein-coupled receptor kinase 6	AAGGATGTTCTGGACATTGAA
IKBKAP	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	CAGCGGTTTACTATAGACAAA
KSR1	kinase suppressor of ras 1	CAAGGCGAAGCTGGTCCGTTA
LMTK3	lemur tyrosine kinase 3	CAAGTTCATCTCGGAAGCACA
MAP3K	mitogen-activated protein kinase kinase	AAGATGGTATATACCAAGTTA

7	kinase 7	
MAP3K7IP2	mitogen-activated protein kinase kinase kinase 7 interacting protein 2	CAGTCAATAGCCAGACCTTAA
MAPKAP1	mitogen-activated protein kinase associated protein 1	CTCCCTTATTCAGGTGGACAA
MARK4	MAP/microtubule affinity-regulating kinase 4	CTGCAGCCTGTTGCCCAATAA
MAST4	microtubule associated serine/threonine kinase family member 4	TTCCCAGTTGTAACCGGTAAA
PIK3C2B	phosphoinositide-3-kinase, class 2, beta polypeptide	CAGGGTGGTCCAGTCCGTCAA
PRKAR1B	protein kinase, cAMP-dependent, regulatory, type I, beta	GACAACGAGAGGAGTGACATA
TAOK2	TAO kinase 2	ACCTACAAACTTCGCAAGGAA
TYRO3	TYRO3 protein tyrosine kinase	AACGGTGACCTTTAGTGCCAA

Table 9: siRNA sequence of the kinases whose knockdown up-regulated *TFF1* expression

Gene	Gene Description	siRNA sequence
BMPR1B	bone morphogenetic protein receptor, type IB	ACGGATATTGTTTCACGATGA
CCRK	cell cycle related kinase	TGGCGAGATAGTTGCCCTCAA
CLK3	CDC-like kinase 3	CACGAAGATCTCGGTCCAGAA
DDR1	discoidin domain receptor family, member 1	ACGGTGTGAATCACACATCCA
DGKZ	diacylglycerol kinase, zeta 104kDa	CAAGAGGAACGACTTCTGTAA
FN3KRP	fructosamine-3-kinase-related protein	TAGGTAGACGGAGCCACACTA
LATS2	LATS, large tumor suppressor, homolog 2 (Drosophila)	AAGGATGTCCTGAACCGGAAT
NEK3	NIMA (never in mitosis gene a)-related kinase 3	ACGATAGAGGTGGTTCTGTAA
NEK8	NIMA (never in mitosis gene a)- related kinase 8	ACGGACAGTTGGGCACCAATA
PANK2	pantothenate kinase 2 (Hallervorden-Spatz syndrome)	CTGTGTGTGAACTTACTGTAA
PIP5K2B	phosphatidylinositol-4-phosphate 5-kinase, type II, beta	CACGATCAATGAGCTGAGCAA
RIOK1	RIO kinase 1 (yeast)	GCCCAACAAGATAATATTCTA
RPS6KA6	ribosomal protein S6 kinase, 90kDa, polypeptide 6	GGCGAGGTAAATGGTCTTAAA

Chapter 3: Results

3.1 Chapter-1

ERα is one of the most studied proteins in breast cancer and is largely responsible for endocrine therapy resistance in breast cancer [305]. miRNAs are small non-coding RNA molecules involved in gene regulation and the modulation of many biological mechanisms. We were interested to explore which miRNAs could be regulated by the estrogen receptor and vice versa. At the outset of this study, no other publications existed regarding regulation of miRNAs by estrogen receptor, but over recent years many studies have revealed the 'cross-talk' between miRNAs and estrogen receptor (which is discussed later in chapter 4.1). We wanted to perform miRNA microarray to reveal all of the possible miRNAs regulated by ERα after the estrogenic response.

3.1.1 GREB1 modulation by ERα in an E2 dependent manner

We wanted to perform a time course experiment after E2 treatment at 3, 6 and 12 hours and evaluate the miRNA expression profile. In order to do so we first carried out initial experiments to optimise the conditions before performing the microarray experiment using GREB1 as a positive control. GREB 1 is a critical regulator of estrogen induced growth of breast cancer cells [306]. To do so, MCF-7 cells were grown in 10% FCS and then switched to a starved condition using DMEM (phenol red free) supplemented with 10% double charcoal stripped FCS (DSS) for 72 hours. After this period the cells were either treated with vehicle or treated with 10nM E2 for the above mentioned time periods. After total RNA extraction from each treated sample, expression of GREB1 was analysed by qRT-PCR using SYBR green and normalized to the GAPDH housekeeping gene,

which remains stable throughout treatment (Fig 12A). We observed the expected fold expression change of *GREB1* at 3h, 6h and 12h of E2 treatment when compared to the untreated sample (vehicle) [306].

As a further control we used JP13 cell lines, MCF-7 cells mutated to conditionally over-expresses a protein composed of the zinc finger transcriptional repressor PLZF fused to ERα (PLZF-ERα) when tetracycline (Tet) is removed from the growth medium (Tet-off system). This fusion protein acts as a dominant-negative that inhibits the expression of estrogen-regulated genes and estrogen-stimulated growth of MCF-7 cells [300]. The JP13 cells were kindly provided to us by Dr. Laki Buluwela.

When compared with the control MCF7-TO cell line (an MCF-7 derived cell line that expresses the tetracycline-regulated transactivator Tet-Off), GREB1 expression was decreased in the JP13 cells when Tet was removed from the medium (Fig. 12B) [307]. These control experiments indicate that treatment with E2 and reduced expression of ER regulated genes with PLZF-ER, these were optimized conditions for producing a miRNA expression profile and identifying ER regulated miRNAs.

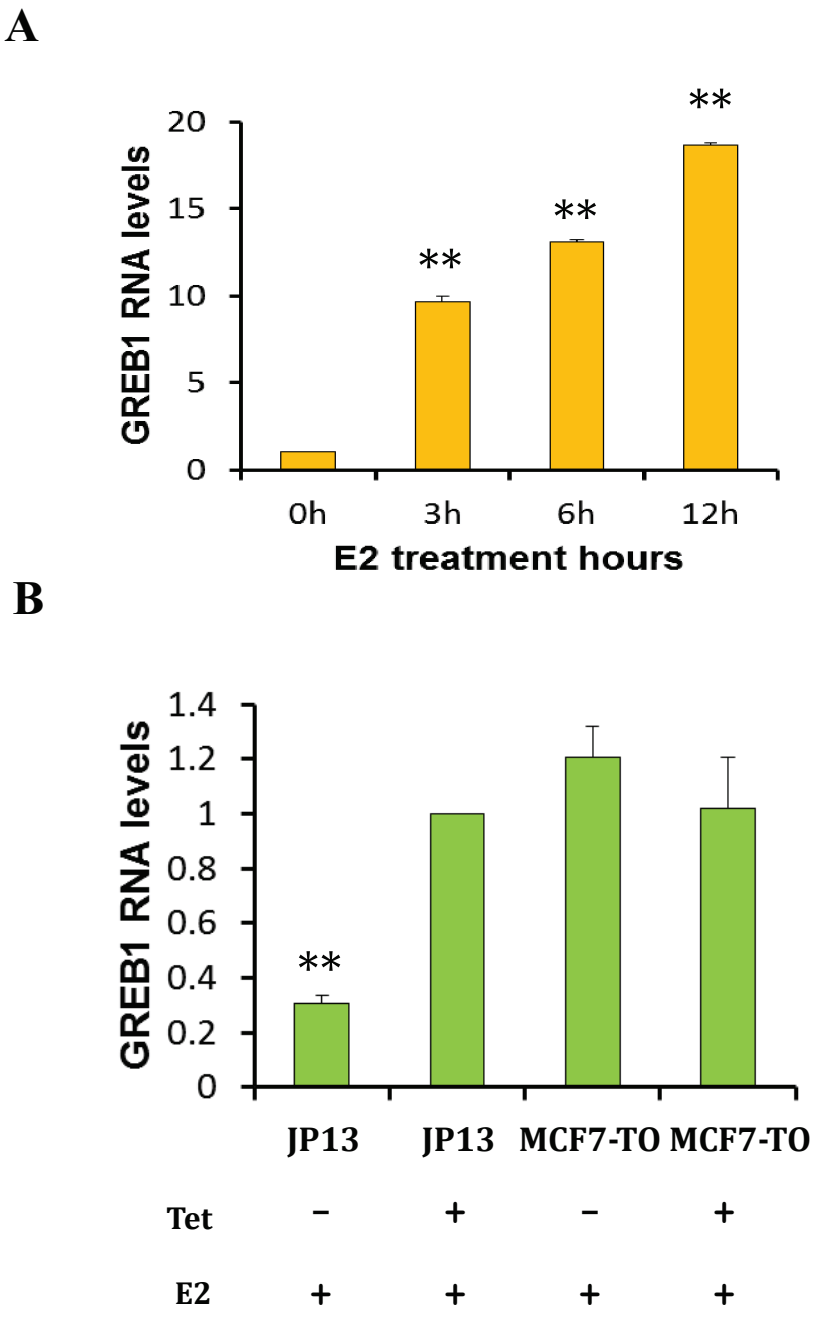


Figure 12: GREB1 is increased by E2 treatment and supressed by over-expression of PLZF-ERα. (A) MCF7 cells were starved for 3 days and then were either left untreated (vehicle – 0h) or treated with 10 nM E2 for time periods (3h, 6h and 12h). After total RNA extraction, expression of GREB1 was analysed and normalized to GAPDH. The mean of three experiments each performed in triplicate are presented, error bars represent s.e.m, ** represents p< 0.005 in comparison to time 0 h. *P* values obtained using a one-tailed Student's *t*-test. (**B**) JP13 and MCF7-TO cells were cultured in the presence or absence of Tet for 72h, followed by the addition of 10nM E2 for 24 h. After total RNA extraction, expression of GREB1 was analysed by qRT-PCR using SYBR green and normalised to GAPDH.

3.1.2 Microarray to reveal ERα regulated miRNAs

To determine whether ERα regulates the expression of miRNAs, MCF-7 cells were maintained in starved condition using DMEM (phenol red free) supplemented with 10 % DSS for 72 hours; then E2 was added at 3h, 6h, 12h respectively as described above, and miRNA chip hybridization was performed to elucidate early (0-3 h) and delayed (>6 h) regulation of miRNAs by ERα. As a control we used the JP13 and MCF-7-TO (MCF-7-Tet-Off) derived cell line. After hybridization, the raw data were imported into the Rosetta Resolver software system for data analysis. Following microarray analysis we did not reveal any miRNAs with expression changes greater than 2 fold comparing 0 h to 3h, 6h and 12 h ($P < 0.05$). Moreover, we found that those miRNAs that increased following E2 in MCF-7 cells (Fig.13A) were decreased in the JP13-Tet-Off system used as a control (Fig.13B). Interestingly, most of the ERα up-regulated miRNAs were derived from mir-17-92 and its paralogue clusters mir-106a-363 and mir-106b-25 (Fig. 8) which are known to be important in cancer biology.

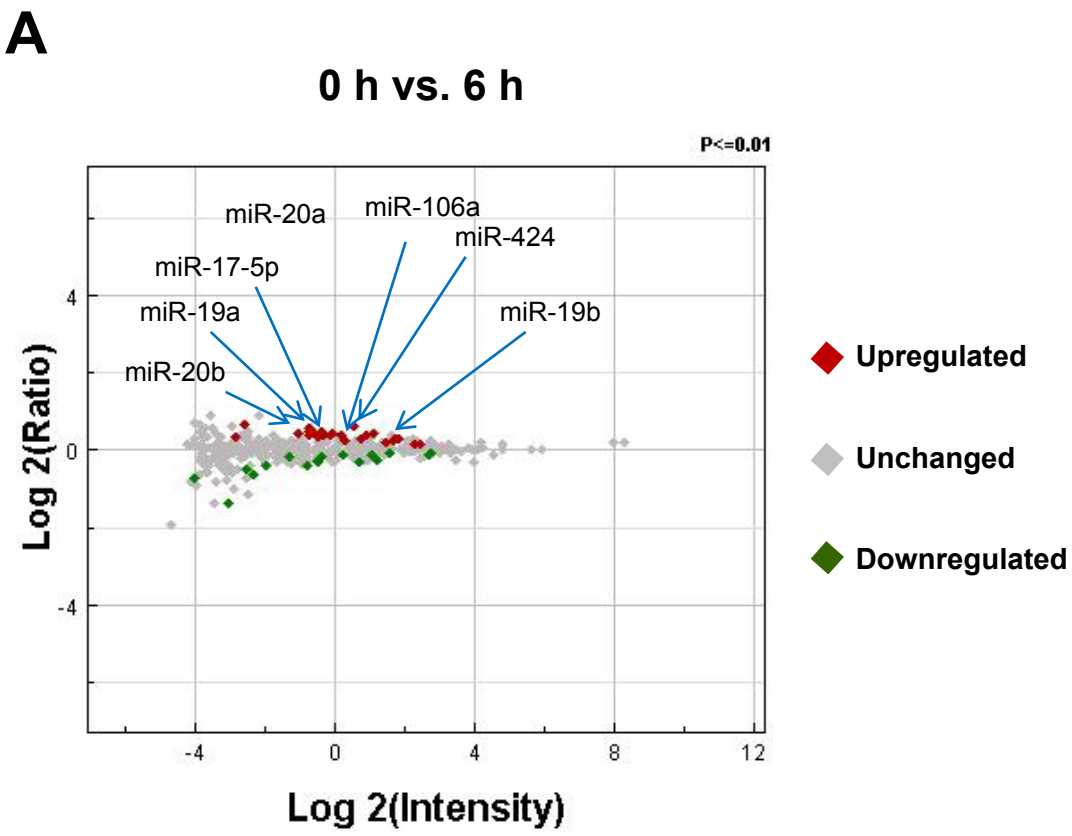


Figure 13: Microarray analysis revealed ERα regulated miRNAs. Graphical representation of upregulated and downregulated genes after 6h of estradiol treatment compared to the vehicle treated. (**A**) MCF-7 cell lines underwent E2 stimulation (10 nM) after 72 h of hormone deprivation. After total RNA extraction and labelling we used a microarray platform containing probes for 470 human miRNAs. After hybridization and scanning, raw data were imported into the Rosetta Resolver system for analysis. $P \leq 0.01$ was used as cut-off for identification of miRNAs up-regulated or down-regulated between 0 h versus 6 h.

B

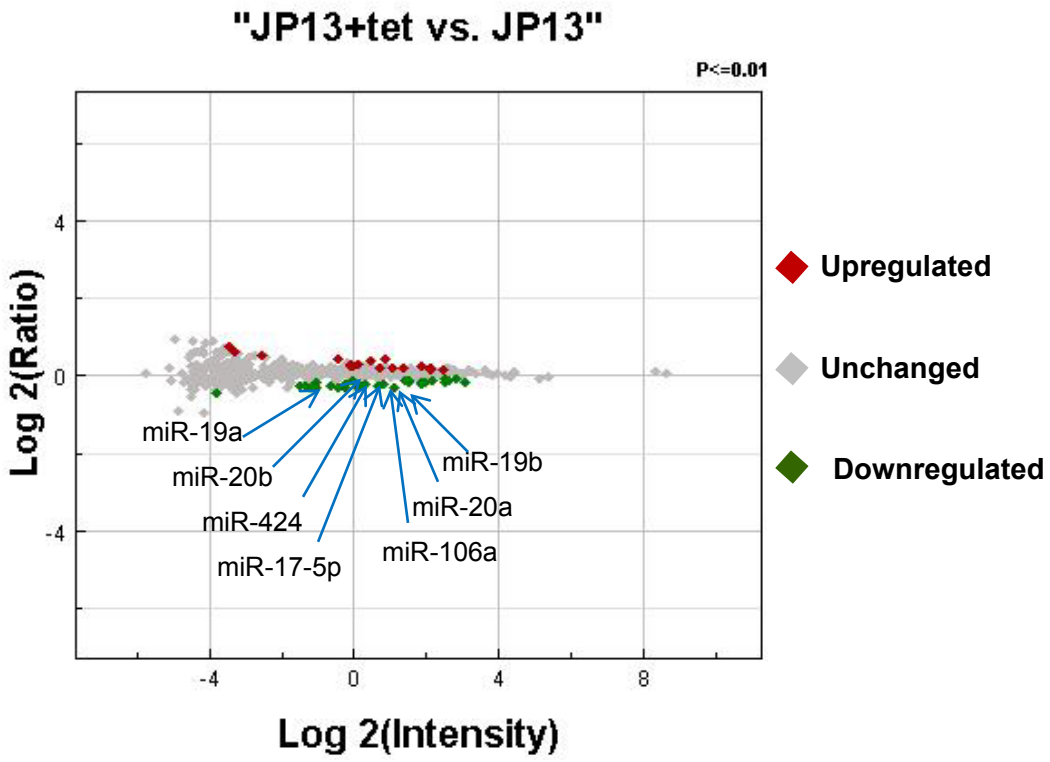


Figure 13: Microarray analysis revealed ERα regulated miRNAs. Graphical representation of upregulated and downregulated genes after 6h of estradiol treatment compared to the vehicle treated in JP13 cells. (**B**) JP13 cells were cultured in the presence or absence of Tet for 72 h, followed by the addition of 10 nM E2 for 24 h prior to microarray analysis. Once again $P \leq 0.01$ was used as cut-off for identification of miRNAs down-regulated in JP13 Tet (+) versus JP13 (Tet-).

Table 10 shows miRNAs up- and down-regulated with significant p-value ($P \leq 0.01$).

miRNA name	Fold change (oh vs 3h)	P value	Fold change (oh vs 6h)	P value	Fold change (JP13+Tet vs jp13)- 6h	P value
hsa-miR-489	1.28	5.15E-03				
hsa-miR-32			1.54	3.28E-03		
hsa-miR-424			1.48	2.00E-05	-1.16	4.77E-03
hsa-miR-101			1.45	1.73E-03		
*hsa-miR-19a			1.36	4.04E-03	-1.26	1.44E-03
hsa-miR-92b					-1.32	4.81E-02
*hsa-miR-20b	1.16	9.13E-02	1.31	9.52E-08	-1.28	5.32E-06
hsa-miR-450			1.24	1.14E-03		
*hsa-miR-19b	1.17	5.69E-03	1.29	5.02E-06	-1.28	2.62E-08
*hsa-miR-20a	1.13	1.55E-02	1.29	1.73E-07	-1.28	4.63E-13
hsa-miR-7	1.23	4.01E-03	1.27	2.59E-14		
*hsa-miR-92					-1.27	2.62E-03
*hsa-miR-106a	1.16	3.95E-03	1.29	3.90E-04	-1.26	4.00E-05
hsa-miR-429			1.26	2.00E-05		
*hsa-miR-17-5p			1.25	4.35E-03	-1.25	6.96E-06
*hsa-miR-18a			1.10	2.78E-01	-1.25	2.46E-07
*hsa-miR-93	1.13	4.46E-03	1.12	1.00E-05	-1.15	3.41E-06
hsa-miR-301					-1.13	1.00E-04
*hsa-miR-25	1.10	2.36E-06	1.15	4.91E-03	-1.14	3.65E-07
*hsa-miR-106b					-1.13	4.21E-06
hsa-miR-181a	-1.11	2.60E-04	-1.10	2.30E-03		
hsa-miR-181b			-1.13	4.58E-03		
hsa-miR-181d			-1.15	9.51E-03		
hsa-miR-181a	-1.36	2.60E-03				
hsa-miR-22	-1.20	1.00E-05				
hsa-miR-30a-5p	-1.24	1.72E-03				
hsa-miR-487b	-1.32	1.26E-03	-1.25	8.56E-03		
hsa-miR-494	-1.27	1.75E-03				
hsa-miR-198			-1.34	3.77E-03		
hsa-miR-500			-1.37	5.87E-03		

Table 10: List of upregulated and downregulated miRNAs from microarray with significant *p-value.* MiRNAs encoded by the 3 paralogous clusters (miR-17–92, miR-106a-363, and miR-106b-25) are indicated by *. In our experiments, for each individual miRNA atleast 2 or 3 probes were spotted on the platform and data are shown for a representative miRNA, but interestingly all the probes abundantly reach our statistical thresholds.

3.1.3 Validation of microarray results by Real-time PCR

To confirm the change of expression detected by the microarray, we performed RT-qPCR from the same RNA samples choosing those miRNAs modulated between 1.2 to 2 fold in cells treated with E2 and those repressed similarly by PLZF-ERα in JP13 cell lines. To validate the results we choose miR-18a, miR-19a and miR-20a from the miR-17-92 cluster and miR-19b, miR-20b and miR-92 from the miR-106a-363 cluster. We also chose miR-424 and miR-181b encoded from different genomic locations, which were up-regulated and down-regulated respectively in the microarray. Finally, we chose miR-342 as negative control as miR-342 levels did not change in the microarray after E2 induction.

RT-qPCR showed that miR-19a (1.5 fold) and miR-20a (1.3 fold) were upregulated although downregulated in JP13 cells (Fig 14b & c). Also, miR-19b (1.5 fold) and miR-20b (1.5 fold) were upregulated but downregulated in JP13 cells (Fig 14d & e). miR-424 was shown to be upregulated (>1.5 fold) and miR-181b was downregulated (0.75 fold). miR-342 (which was used as negative control) did not change with either estradiol treatment or in JP13 cells. Therefore, RT-qPCR showed comparable results to those obtained from the microarray, reaching significance ($P<0.05$) in most cases. These results confirm the precision of the miRNA profiling results from the microarray experiment.

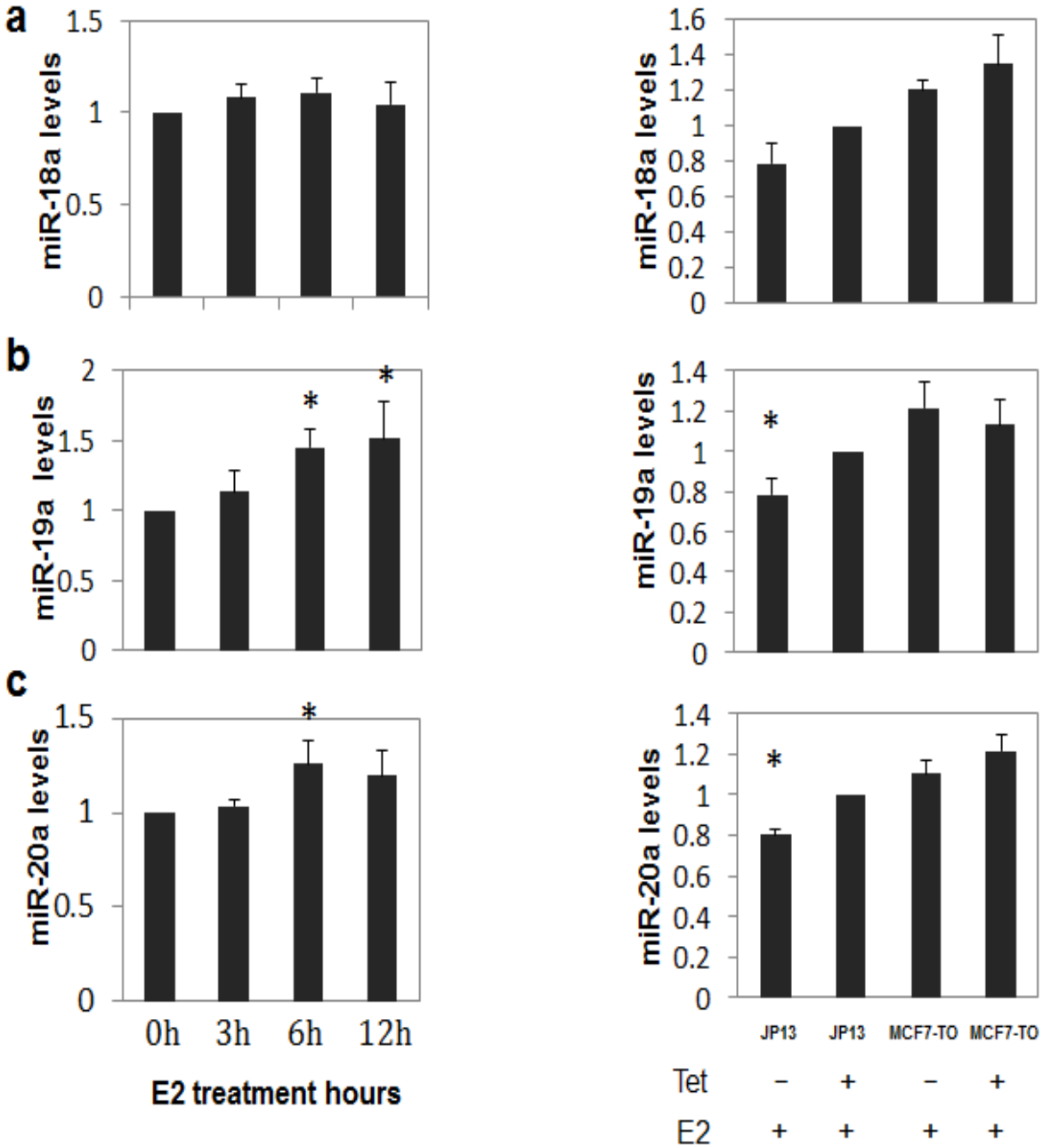


Figure 14: Validation of microarray results by qRT-PCR. miRNAs up or down regulated were validated by Taqman qRT-PCR after 0h, 3h, 6h and 12 h of E2 treatment and after PLZF-ERα over-expression **(a)** miR-18a **(b)** miR-19a **(c)** miR-20a

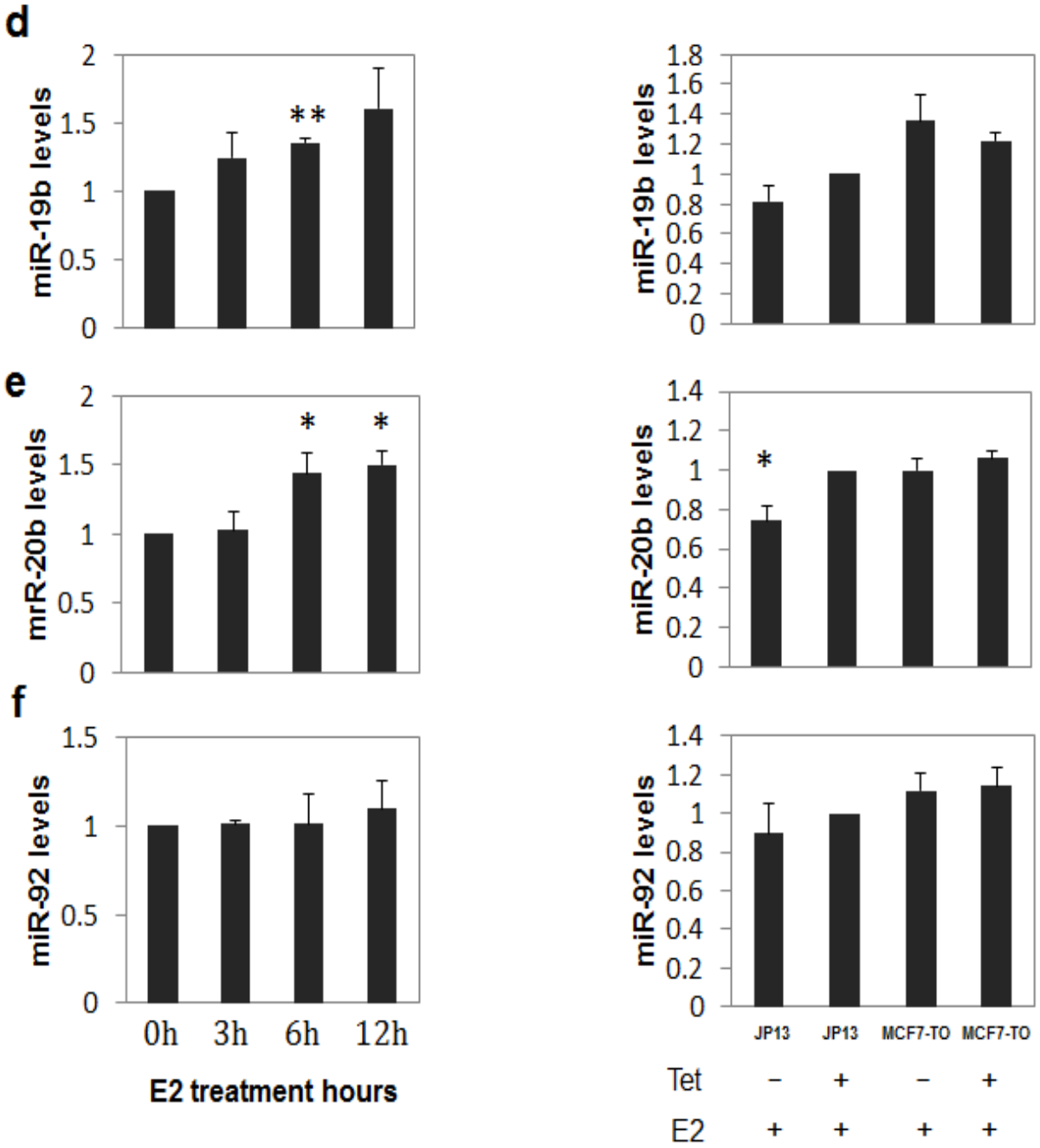


Figure 14: Validation of microarray results by qRT-PCR. miRNAs up or down regulated were validated by Taqman qRT-PCR after 0h, 3h, 6h and 12 h of E2 treatment and after PLZF-ERα over-expression (**d**) miR-19b (**e**) miR-20b (**f**) miR-92.

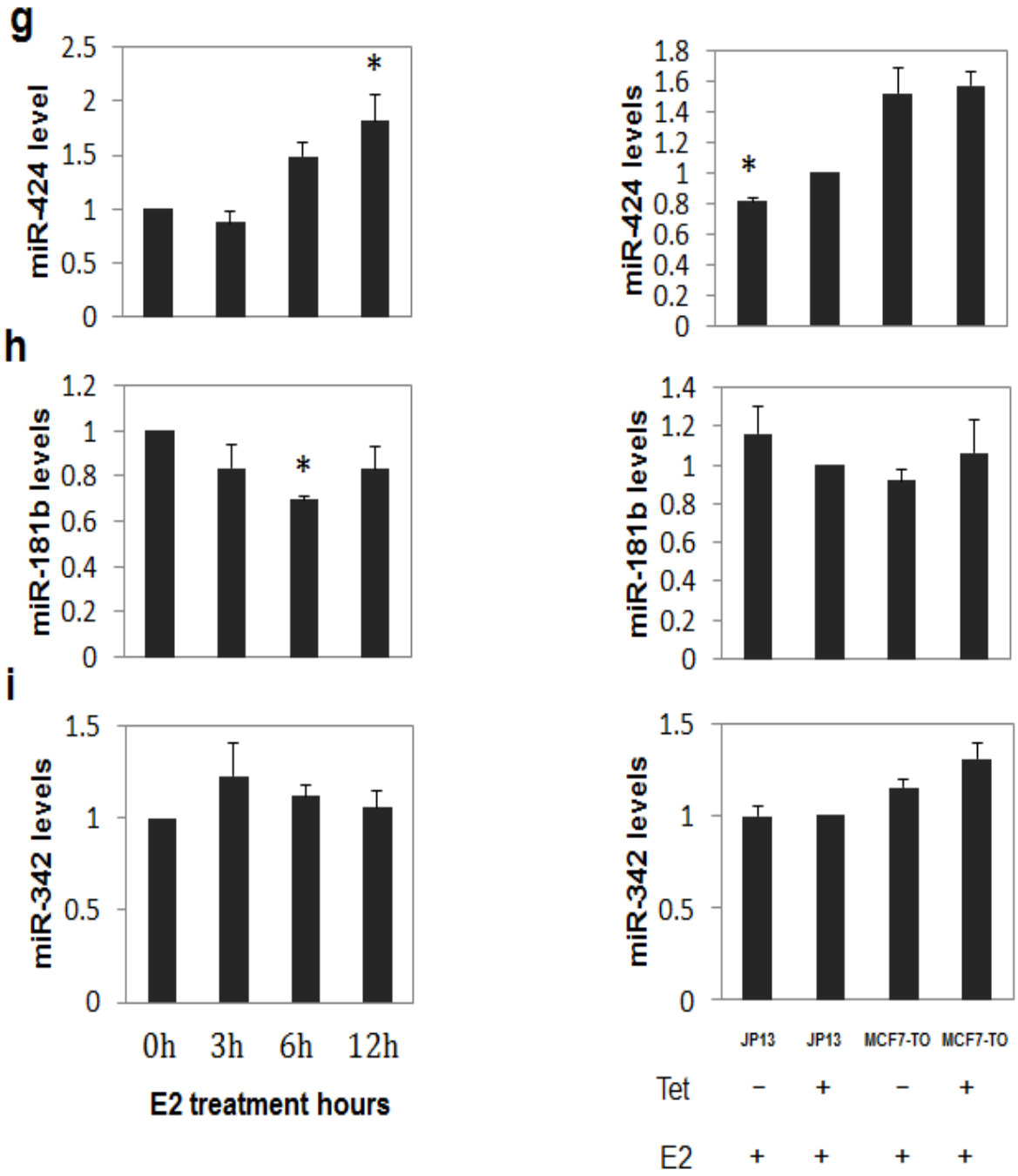


Figure 14: Validation of microarray results by qRT-PCR. miRNAs up or down regulated were validated by Taqman qRT-PCR after 0h, 3h, 6h and 12 h of E2 treatment and after PLZF-ERα over-expression (**g**) miR-424, (**h**) miR-181b and (**i**) miR-342 which was used as negative control. The mean of three experiments each performed in triplicate are presented, error bars represent s.e.m. * represents $P<0.05$ in comparison to time 0 h, ** represents $P<0.005$. P value obtained using a one-tailed Student's t-test.

3.1.4 ERα induction reveals pri-mir-17-92 up-regulation

We further went on to examine the expression of the unprocessed pri-mir-17-92 and family members using RT-qPCR. Pri-mir-17-92 appeared up-regulated within 3h of E2 treatment reaching a 4-5 fold change in comparison to 0h (Fig. 15A) defining it as a new early ERα up-regulated gene. As expected, their expression levels were significantly repressed by PLZF-ERα (Fig. 15B). Pri-mir-342, a negative control showed no changes (Fig. 15A & B)

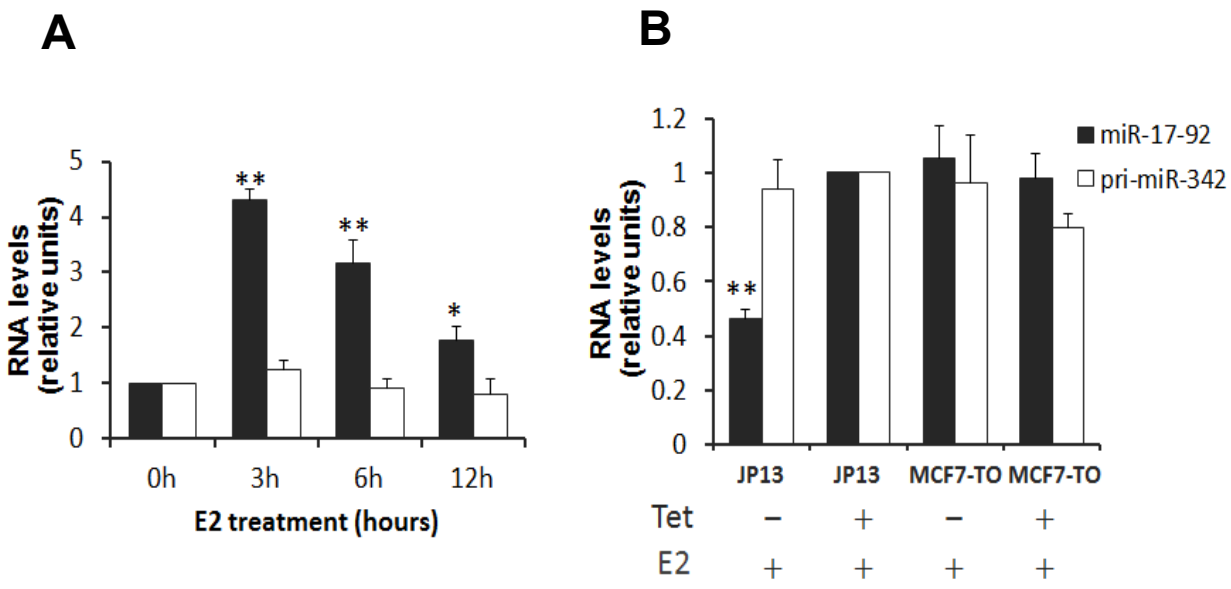


Figure 15: Pri-mir-17-92 is increased by E2 and decreased by over-expression of PLZF-ERα. (A) MCF-7 cells were maintained in DMEM (minus phenol red) supplemented with 10 % charcoal-dextran FBS for 3 days and then were either left untreated or treated with 10 nM E2 for the indicated time periods. After total RNA extraction, expression of pri-mir-17-92 and pri-miR-342 was analyzed by RT-qPCR using SYBR green and normalized to GAPDH. (**B**) JP13 and MCF-7-TO cells were cultured in the presence or absence of Tet for 72 h, followed by the addition of 10 nM E2 for 24 h. Once again, after total RNA extraction, expression of pri-mir-17-92 and pri-miR-342 were analyzed by RT-qPCR using SYBR green and normalized to GAPDH. The mean of three experiments each performed in triplicate are presented, error bars represent s.e.m. For RT-qPCR data, the asterisk indicates $P < 0.05$ in comparison to time 0 h, the double asterisk represents $P < 0.005$ in comparison to time 0 h. *P* values obtained using a two-tailed Student's *t*-test.

We obtained the same results normalizing the value of expression for several housekeeping genes (GAPDH, snRNA U6 and snRNA U47) (Fig. 16 A&B).

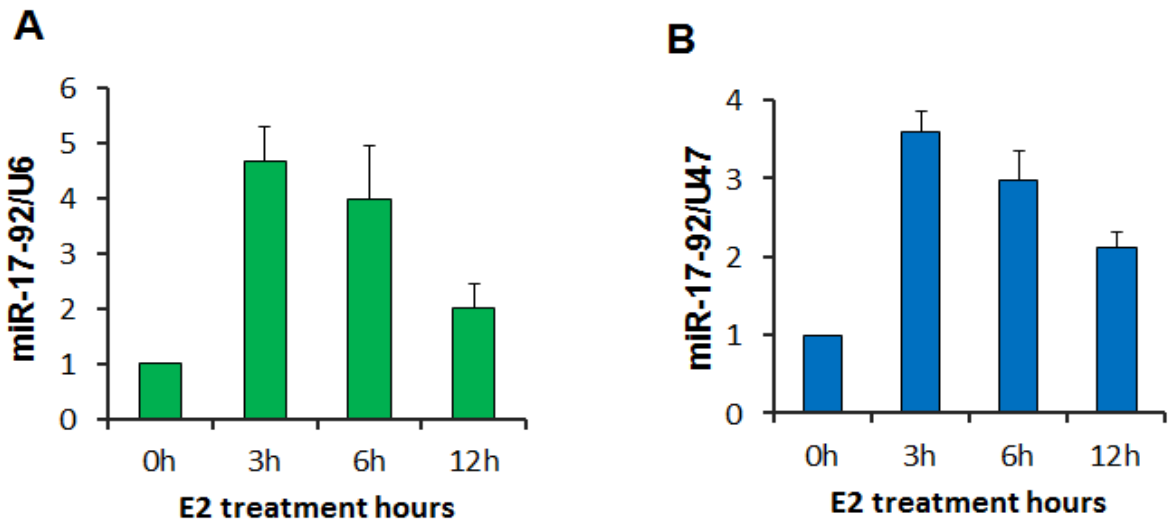


Figure 16: miR-17-92 expression levels after E2 treatment normalized by two housekeeping small RNAs. After MCF7 hormone starvation and treatment with 10 nM of E2 for 0h, 3h, 6h and 12h, expression of miR-17-92 was analysed by qRT-PCR. (**A**) miR-17-92 expression levels normalized to U6. (**B**) miR-17-92 expression levels normalized to U47. Average of three experiments each performed in triplicate are presented, error bars represent s.e.m.

3.1.5 Pri-mir-17-92 is negatively regulated following DROSHA cleavage delaying miRNA maturation over time

Remarkably, the pri-mir-17-92 expression were strikingly upregulated compared to the miRNAs that are produced by its processing (miR-17, miR-18a, miR-19a, miR-19b and miR-20a), indicative of modulation of miRNAs biogenesis at the post-transcriptional level (Fig. 15A and 17A). A primary transcript undergoes a dual processing event, the first in the nucleus by DROSHA (pre-miRNA production), the second in the cytoplasm by DICER. In order to define the steps of miRNA biogenesis in which this regulation occurs, we measured levels of the pri-miR-17-92 derived pre-miR-18a after E2 treatment. DROSHA pri-mir-17-92 cleavage to pre-miR-18a was not a regulatory or 'rate-limiting' step here because both were induced at similar levels (Fig. 17B).

These data demonstrate that pri-mir-17-92 is induced by the E2-ERα complex, then it is processed by DROSHA releasing the pre-miR-18a, but the passage between pre-miR-18a and miR-18a is blocked until at least 12h following initial E2 stimulation. Furthermore, using RT-qPCR, we found that both miR-18a and miR-20a mature forms increase their levels of expression from 24 - 72 h after E2 stimulation (Fig. 17C & D). Northern blot analysis also confirmed that miR-18a increase their levels of expression from 24 - 72 h after E2 stimulation (Fig. 17E). Analys ing the levels of the pri-mir-17-92 and the pre-miR-18a from 0 - 72h, we observed that pri-mir-17-92 is transcriptionally up-regulated after 3h, then DROSHA promptly processes the pri- to the pre-miR-18a, whereas the formation

of the mature form from the pre-miR-18a is delayed (Fig. 17F). In addition while the miR-18a levels start to increase at 24 h, both pri-mir-17-92 and pre-miR-18a levels decline, indicative of the processing delay we observed (Fig. 17F).

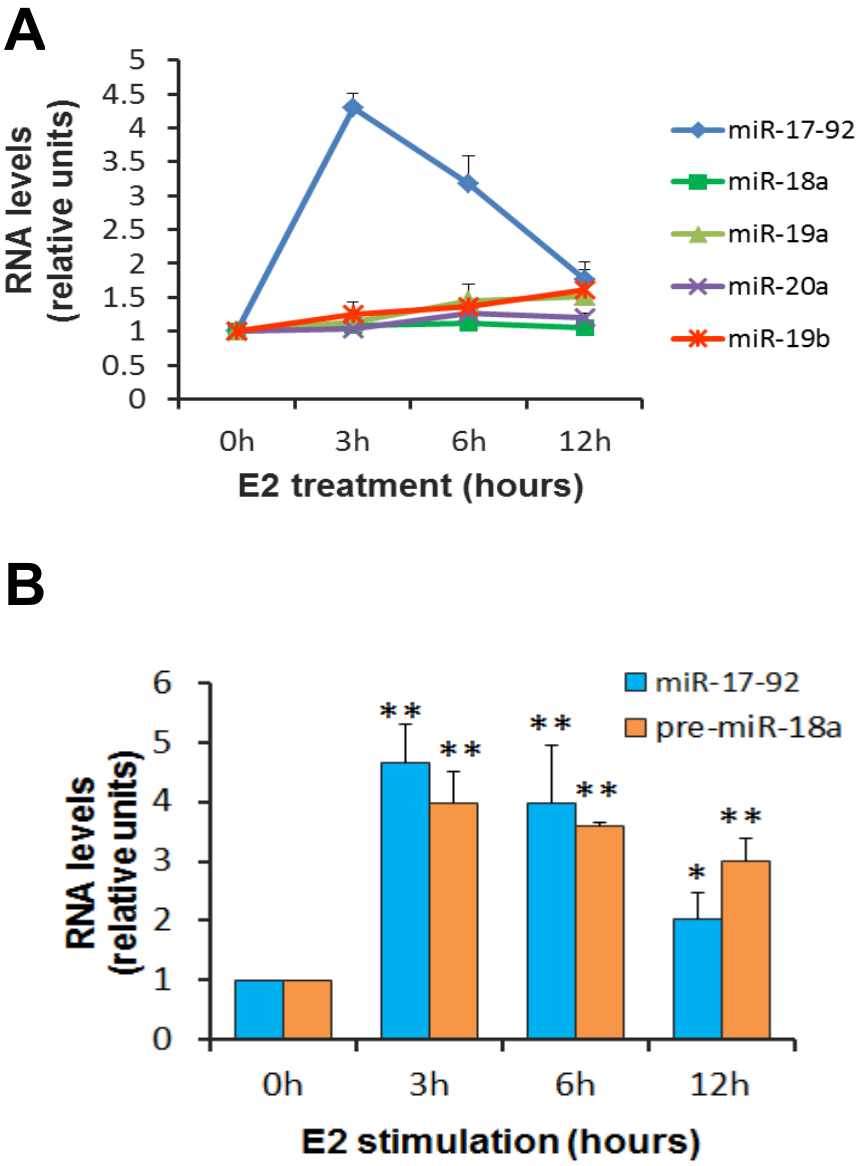


Figure 17: Pri-mir-17-92 is negatively regulated following DROSHA cleavage delaying miRNA maturation over time. (A) Comparison of the levels of expression between pri-mir-17-92 (normalized to GAPDH) and miRNAs encoded from this cluster (normalized to U47). The mean of three experiments each performed in triplicate are presented, error bars represent s.e.m. **(B)** After starvation, expression levels of both pri-mir-17-92 and pre-miR-18a have been analysed by RT-qPCR using SYBR green and normalized to U6 snRNA following E2 treatment as indicated. The mean of three experiments each performed in triplicate are presented, error bars represent s.e.m. * indicates $P < 0.05$ in comparison to time 0 h, ** represents $P < 0.005$ in comparison to time 0 h. *P* values obtained using a two-tailed Student's *t*-test.

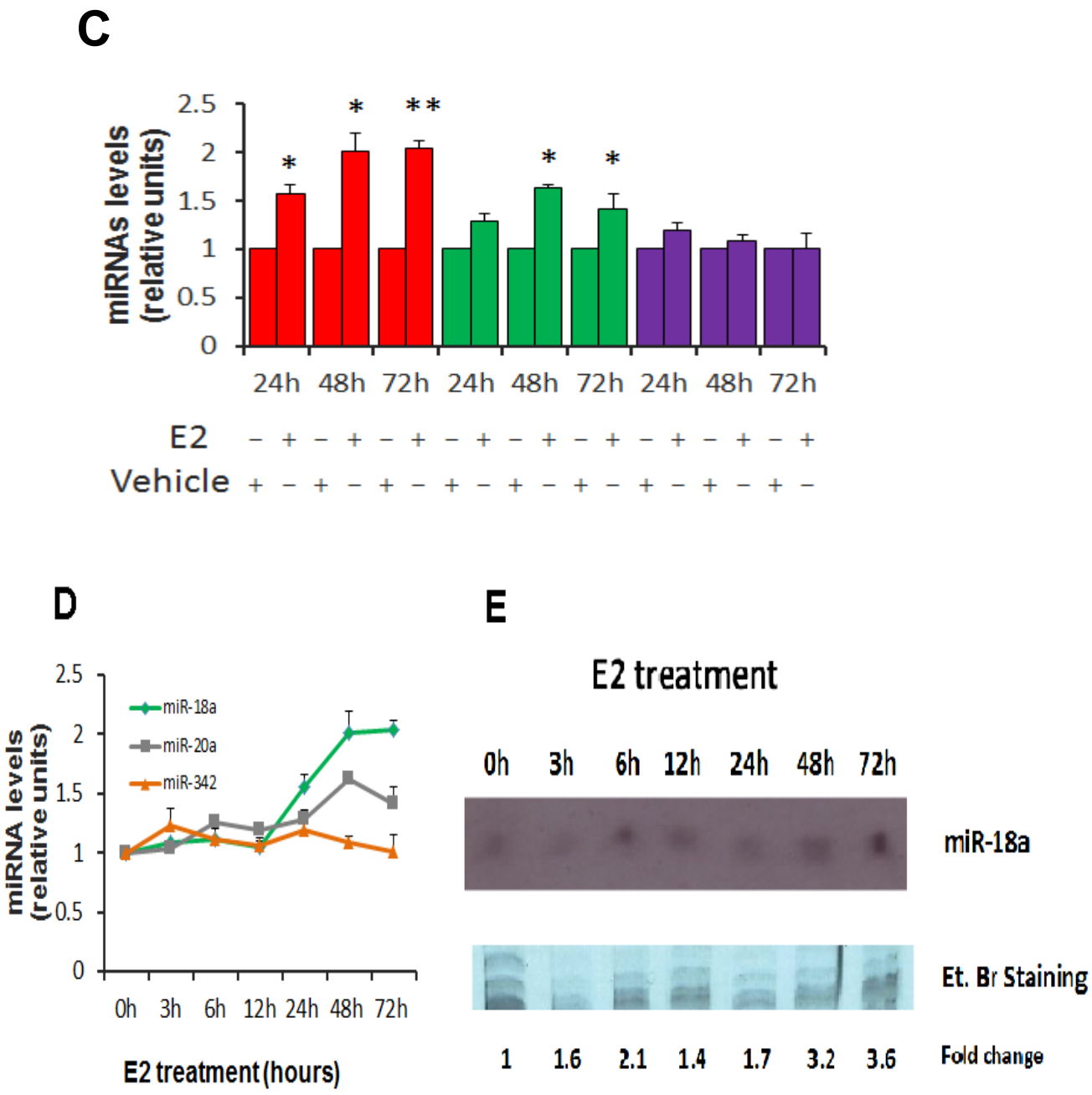


Figure 17: miR-18a, miR-20a and miR-342 levels after estradiol treatment. **(C)** After starvation expression levels of miR-18a, miR-20a and miR-342 was analysed by RT-qPCR and normalized to U47 snRNA. The mean of three experiments each performed in triplicate are presented, error bars represent s.e.m. * indicates $P < 0.05$ in comparison to vehicle treatment; ** represents $P < 0.005$ in comparison to vehicle treatment. *P* values obtained using a two-tailed Student's *t*-test. **(D)** Representation of miR-18a, miR-20a and miR-342 levels (normalization to U47 snoRNA) from 0 to 72 h of E2 treatment by RT-qPCR. The mean of three experiments each performed in triplicate are presented, error bars represent s.e.m. **(E)** Northern blotting of miR-18a from 0 to 72 h of E2 treatment. The values represent densitometric scanning of the miR-18a bands normalized to the loading levels (Ethidium bromide staining of the small RNA fraction).

F

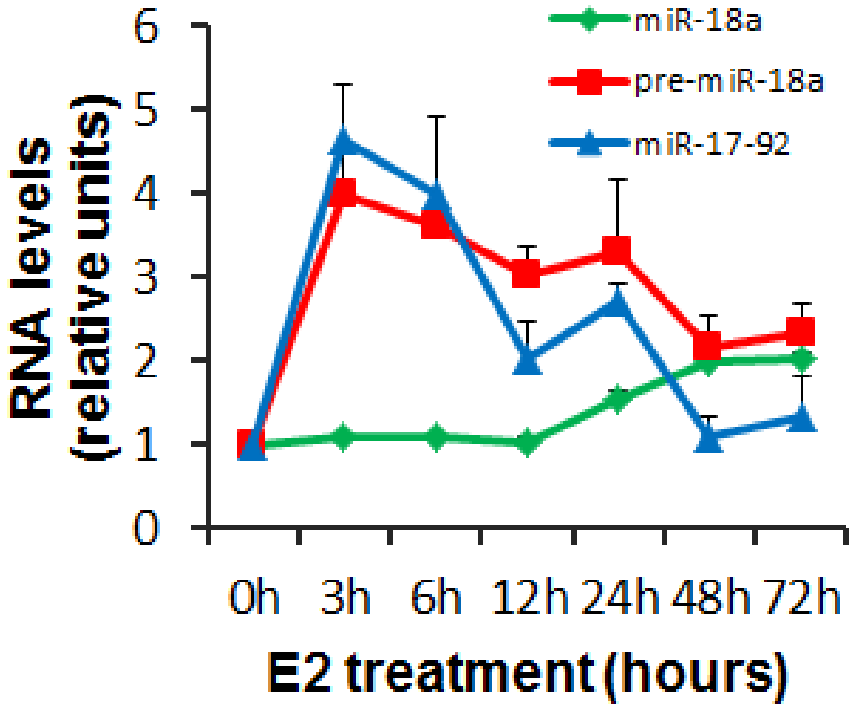


Figure 17: Levels of miR-17-92, pre-miR-18a and miR-18a after estradiol treatment. (F) Representation of the miR-18a (normalization to U47), pre-miR-18a and pri-mir-17-92 levels (normalization to U6) from 0 to 72 h of E2 treatment. The mean of three experiments each performed in triplicate are presented, error bars represent s.e.m.

The primers used to amplify the pre-miR-18a would also amplify pri-mir-17-92. Therefore, to show that we could distinguish between pri- and pre-miRNA, we stimulated the cell lines with E2 and then separated the small RNA fraction from the large RNA fraction. We used the large RNA fraction to measure pri-mir-17-92 and the small RNA fraction to measure pre-miR-18a (Fig. 18). As a further control we measured the pri-mir-17-92 from the small RNA fraction without obtaining any amplification product.

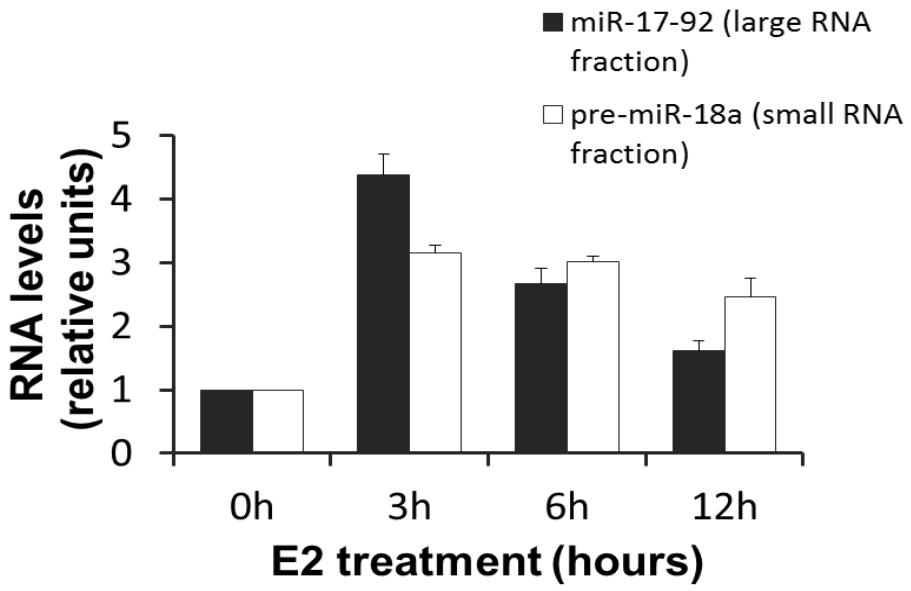


Figure 18: Pri- miR-17-92 is promptly processed by DROSHA complex that releases pre-miR-18a. After MCF7 hormone starvation and treatment with 10 nM of E2 for 0h, 3h, 6h and 12h, RNA fractions were separated and used for qRT-PCR. MiR-17-92 expression levels from large RNA fraction (>200 nt), normalized to GAPDH levels are presented with white columns, whereas pre-miR-18a expression levels from small RNA fraction (<200 nt), normalized to U6 are presented with dark columns. Average of three experiments each performed in triplicate are presented, error bars represent s.e.m.

Cycloheximide is an inhibitor of protein biosynthesis and exerts its effect by interfering with translational elongation during the translocation step therefore blocking protein synthesis [308]. Using cycloheximide (CHX), we demonstrated that new protein synthesis is not required exclusively for pri-mir-17-92 expression.

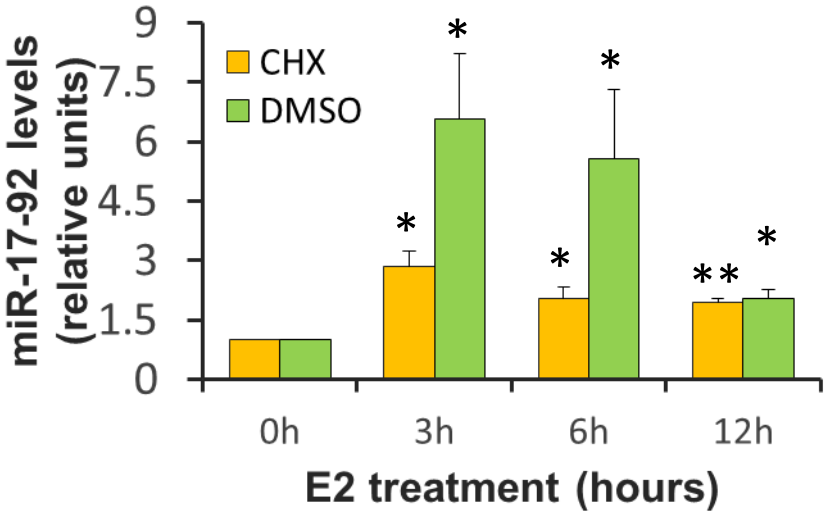


Figure 19: New protein synthesis is not required exclusively for E2-induced mir-17–92 expression. MCF-7 cells were maintained in DMEM (minus phenol red) supplemented with 10% charcoal-dextran FBS for 3 days and then pretreated with either CHX or DMSO (vehicle) as indicated, 1h before 10 nM E2 treatment. After total RNA extraction, the expression of mir-17–92 was analysed by qRT-PCR using SYBR green and normalized to GAPDH. The mean of 3 experiments each performed in triplicate are presented, error bars represent SEM. * indicates $P<0.05$ in comparison to time 0h, ** represents $P<0.005$ in comparison to time 0h. P values were obtained using a 2-tailed Student's t-test.

3.1.6 C-MYC directly regulates the pri-mir-17-92 upon estrogenic stimulation

It has already been proven that pri-mir-17-92 is transcriptionally regulated by c-MYC in the P493-6 B cell line during the G1-S cellular transition phase [153]. Since c-MYC mRNA is up-regulated by ERα within 1 h of E2 treatment in breast cancer cells [309], c-MYC could contribute to the increased transcription of the pri-mir-17-92 upon E2 stimulation. Interestingly, we observed a half site conserved estrogen response element (ERE) 70 bp upstream of the c-MYC consensus site (E-box) of the mir-17-92 promoter (Fig. 20).

Figure 20: Putative ERE and c-MYC consesus elements in mir-17–92 promoter are located in close proximity and conserved among species. Alignment between human, mouse, and rat of the genomic sequence of mir-17–92 promoter, containing both a putative half-site ERE and the E-box (c-MYC binding site).

The putative half-site ERE is indicated in red, the E-box in green. Since it has been demonstrated that estrogen responsive genes can contain both ERα and c-MYC binding elements located within close proximity (13-214 bp) of the promoter and regulated by both transcription factors in a E2 dependent manner [310], we performed chromatin immunoprecipitation (ChIP) assays for both ERα and c-MYC, and co-precipitated DNA was analysed by amplifying the genomic region containing both consensus sites (Fig. 21A) by real time PCR (Fig. 21B & C). Although TFF1, a known estrogen regulated gene, is confirmed here as regulated by ERα (Fig. 21B), we observed only c-MYC interacting with the mir-17-92 promoter region analysed (Fig. 21B & C). We demonstrated that c-MYC is recruited to the mir-17-92 promoter in breast cancer cells upon E2 stimulation.

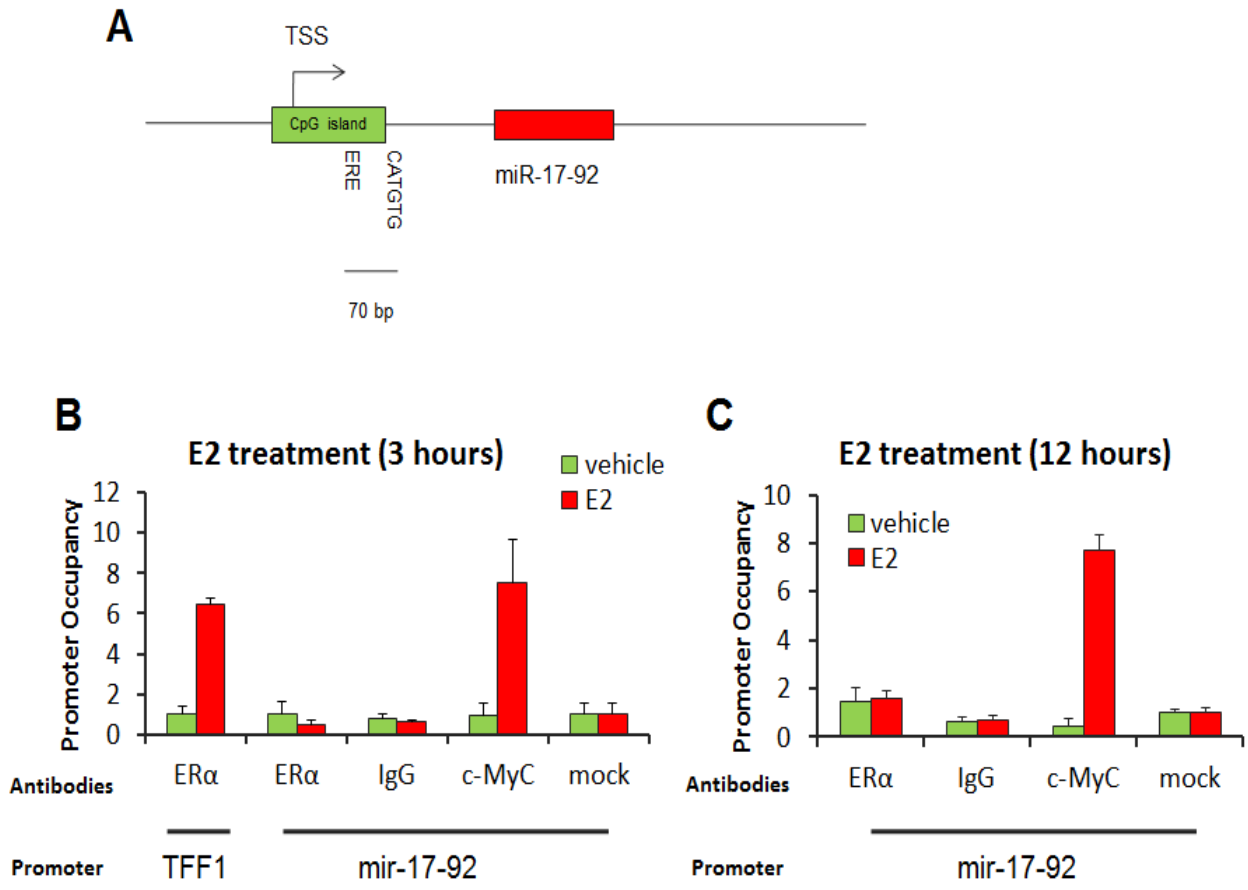


Figure 21: c-MYC directly regulates the pri-mir-17-92 upon estrogenic stimulation. (A) Schematic representation of the mir-17-92 cluster genomic region. Both the c-MYC binding site and a putative ERE half site are indicated. **(B)** MCF-7 cells were maintained in estrogen-free medium for 3 days (starvation) and then either left untreated (vehicle) or treated with 10 nM E2 for 3 h after which ChIP was performed, followed by real time PCR. The c-MYC interaction site genomic region is presented. **(C)** After starvation, MCF-7 cells were treated with E2 for 12 h prior to ChIP.

3.1.7 Pri-mir-17-92 expression is correlated with ERα levels in ERα-positive primary breast cancers

To evaluate ERα modulation of the pri-mir-17-92 at the physiologic level, we examined a correlation between ERα mRNA and pri-mir-17-92, and ERα mRNA and pre-miR-18a, in breast cancer tissues by RT-qPCR. Levels of pri-mir-17-92 were correlated with ERα mRNA in tissues (r^2 = 0.97, P = 0.0002, Fig. 22A), further indicating that ERα regulates the expression of this primary miRNA. However pre-miR-18a was less well correlated with ERα (r^2 = 0.54, P = 0.21, Fig. 22B). Next, we addressed whether pre-miR-18a and miR-18a were differentially expressed in primary breast cancer tissues, comparing the average expression levels between ERα-positive and negative tumours. Pre-miR-18a levels were significantly higher in ERα-positive tumours (2.52 $\pm$ 0.30) compared with negative tumours (0.90 $\pm$ 0.08, P = 0.006, Fig. 22C) supporting our data. Moreover, expression levels of miR-18a showed no significant difference between the two groups of samples (Fig. 22D), indicating that impaired pre-miR-18a processing to miR-18a occurs in tumours.

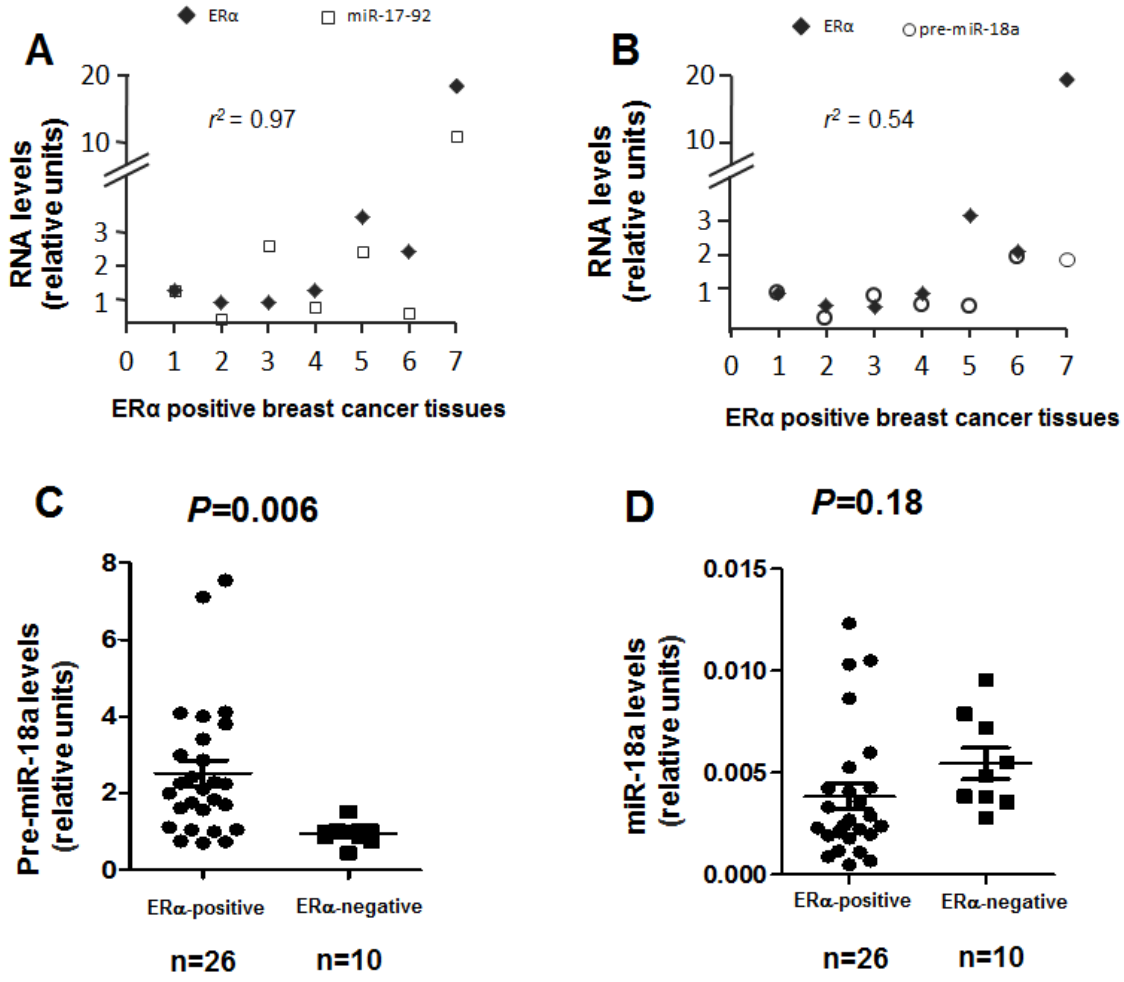


Figure 22: ERα modulates pri-mir-17-92 in breast cancer tissues. (**A**) Expression levels of ERα and pri-mir-17-92 (Pearson correlation 0.97) or (**B**) pre-miR-18a (Pearson correlation 0.54) was measured by RT-qPCR in ERα-positive breast cancers. (**C**) RT-qPCR showed that expression levels of pre-miR-18a is significantly higher in ERα-positive then in ERα-negative tumours (unpaired, two-tailed Student's *t*-test $P = 0.006$). Error bars represent s.e.m. (**D**) RT-qPCR showed that expression levels of miR-18a are not different between ERα-positive and ERα-negative tumours (unpaired, two-tailed Student's *t*-test $P = 0.18$). Error bars represent s.e.m.

3.1.8 miR-18a, miR-20b and miR-19b negatively modulate the ERα transcriptional activity after estrogen stimulation

Using available miRNA target prediction software (TargetScan [311], miRBase [312], Pictar [313] and Pita [314]), we looked to see if ERα is a potential target of some or all of these miRNAs.

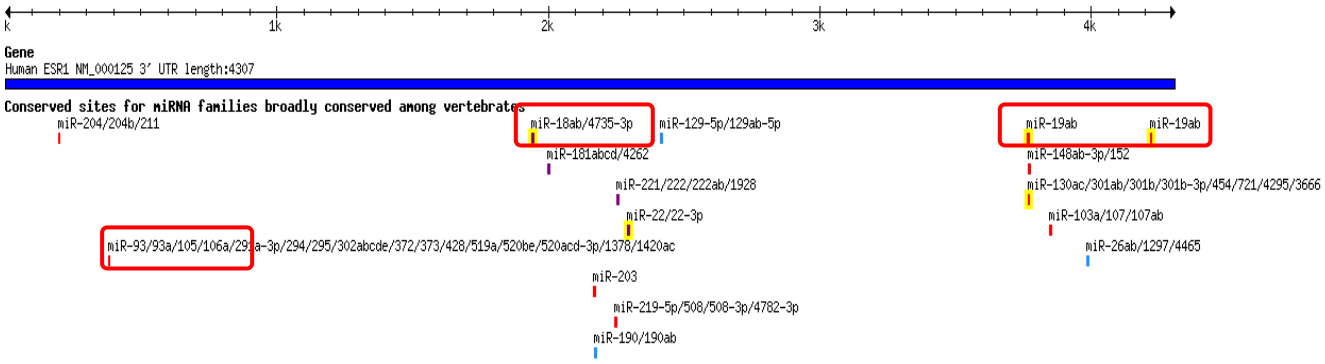


Figure 23: Prediction of the miRNAs targeting 3'UTR of ERα using targetscan. miRNAs targeting ERα from mir-17-92 and paralogue clusters have been highlighted in red rectangles.

Surprisingly, we found that miR-18, miR17/20/106 and miR-19 family members were predicted to target ERα (Fig. 23 & Fig.24A). To experimentally validate this prediction, we chose miR-18a encoded by pri-mir-17-92, miR-19b encoded by both pri-mir-17-92 and the pri-mir-106a-363, and miR-20b encoded by the pri-mir-106a-363. First, we assessed whether these miRNAs influence ERα transcriptional activity. MELN cells (MCF-7 cells, stably transfected with a luciferase reporter gene under the control of an ERE using the β-globin promoter) were transfected with pre-miR-18a, pre-miR-20b and pre-miR-negative control (pre-miR-n.c). E2 stimulated reporter activity was significantly reduced when MELN cells were transfected with pre-miR-18a and pre-miR-20b, whereas the level of induction was not affected by pre-miR-n.c. (Fig. 24B). Remarkably, anti-miR-18a, anti-miR-20b and anti-miR-19b molecules able to silence their miRNA function, significantly increased reporter activity (Fig. 24C). The effect of miRNA silencing on luciferase reporter activity was similar to treatment with anti-miR-17-5p, previously reported to reduce the transcriptional activity of ERα by down-regulating the co-activator AIB1 [171] (Fig. 24C).

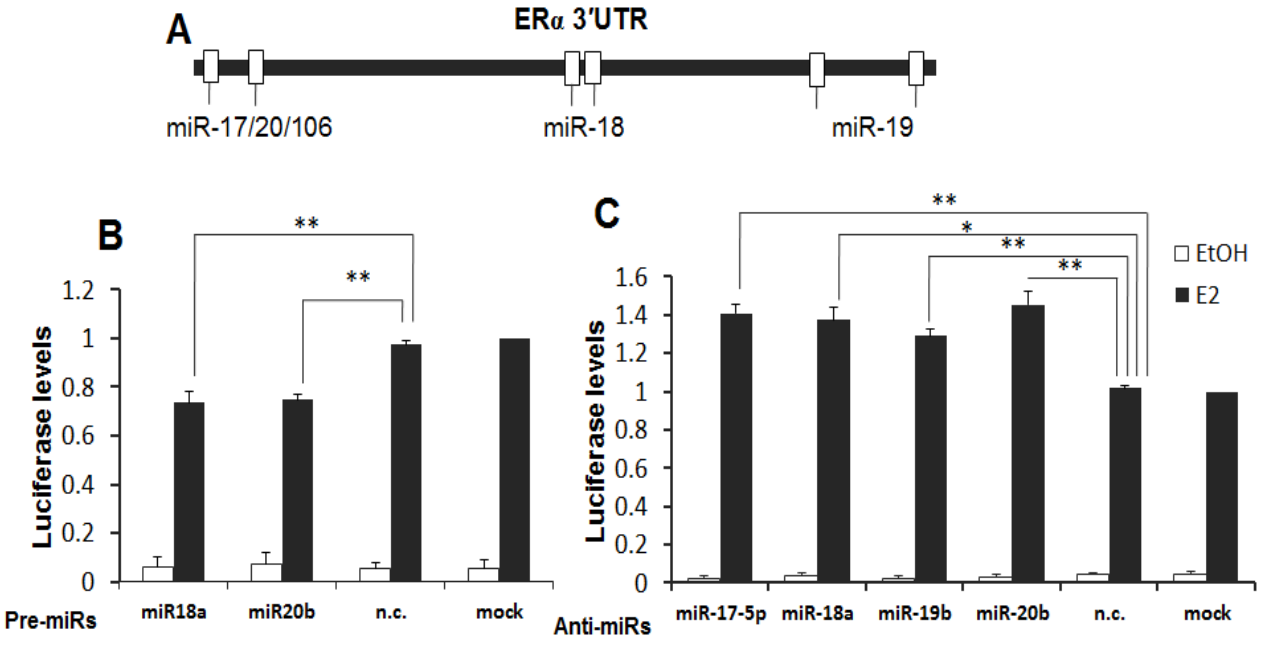


Figure 24: miR-18a, miR-19b and miR-20b suppress ERα mediated signalling. (A) MiRNAs predicted to target the ERα 3'UTR using target prediction software. **(B)** Luciferase activity in MELN cells untransfected or transiently transfected for 48 h with pre-miR-18a, pre-miR-20b and pre-miR-n.c in the absence or presence of 10 nM of E2 for 24h **(C)** Luciferase activity in MELN cells transiently transfected for 48 h with anti-miR-17-5p, anti-miR-18a, anti-miR-19b, anti-miR-20b, anti-miR-n.c or untransfected in the absence or presence of 10 nM of E2 for 24h.

3.1.9 miR-18a, miR-20b and miR-19b suppresses ERα and its co-activator AIB1 at the translational level

The results presented so far indicate that any of the composer or ER transcriptional machinery could be directly targeted by these miRNAs. mir-17-5p, miR-106b and miR-20a are able to negatively regulate AIB1 protein translation by a direct interaction with the 3'UTR of AIB1 mRNA [171, 172, 174]. Since we observed that miR-17/20/106 and the miR-18 family members potentially target ERα, we evaluated if the reduction in ERα transcriptional activity induced by miR-20b over-expression was due to the contemporary negative regulation of AIB1 and ERα. In addition, if the reduction in ERα transcriptional activity induced by over-expression of miR-18a was due to a reduction of ERα protein levels.

In order to address if these miRNAs negatively regulate either ERα and/or its co-activator partner AIB1, we over-expressed pre-miR-18a, pre-miR-19b, pre-miR-20b and pre-miR-n.c. and measured their protein levels. Notably, ERα was markedly reduced by the over-expression of all three pre-miRs analysed in comparison to either untransfected or pre-miR-n.c transfected cells although the reduction with pre-miR-19b was less pronounced (Fig. 25A & B). Furthermore, miR-20b reduced AIB1 protein levels (Fig. 25A & C).

We further transfected pre-miR-18a, pre-miR-19b, pre-miR-20b, and pre-miR-n.c for 48h and checked RNA levels of ERα, AIB1 and also the ER co-activator TIF2. We did not observe reduction for either ERα or AIB1 mRNA levels after transfection of these precursors, suggesting that these miRNAs regulate these genes at the protein translation step (Fig. 26A, B &C).

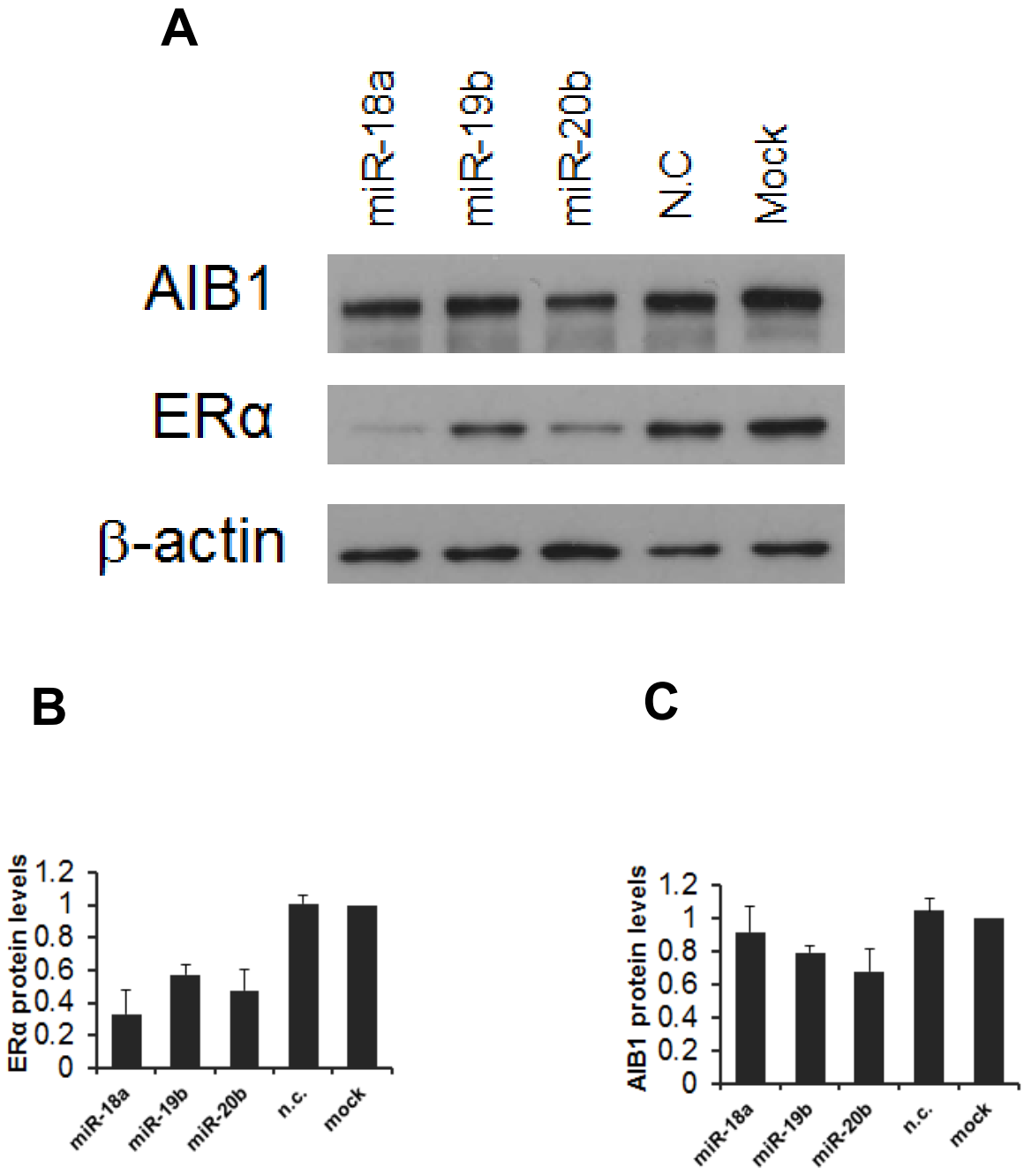


Figure 25: ERα and AIB1 protein levels reduces upon pre-miRNA overexpression. (A) Western blot showing ERα, AIB1 and β-actin in MCF-7 cells untransfected or transiently transfected with pre-miR-18a, pre-miR-19b, pre-miR-20b and pre-miR-n.c. **(B)** Densitometric analysis of ERα Western blot (shown in panel A) (**C**) and Densitometric analysis of AIB1 Western blot (shown in panel A), both normalized to β-actin. The mean of three independent experiments are presented, error bars represent s.e.m.

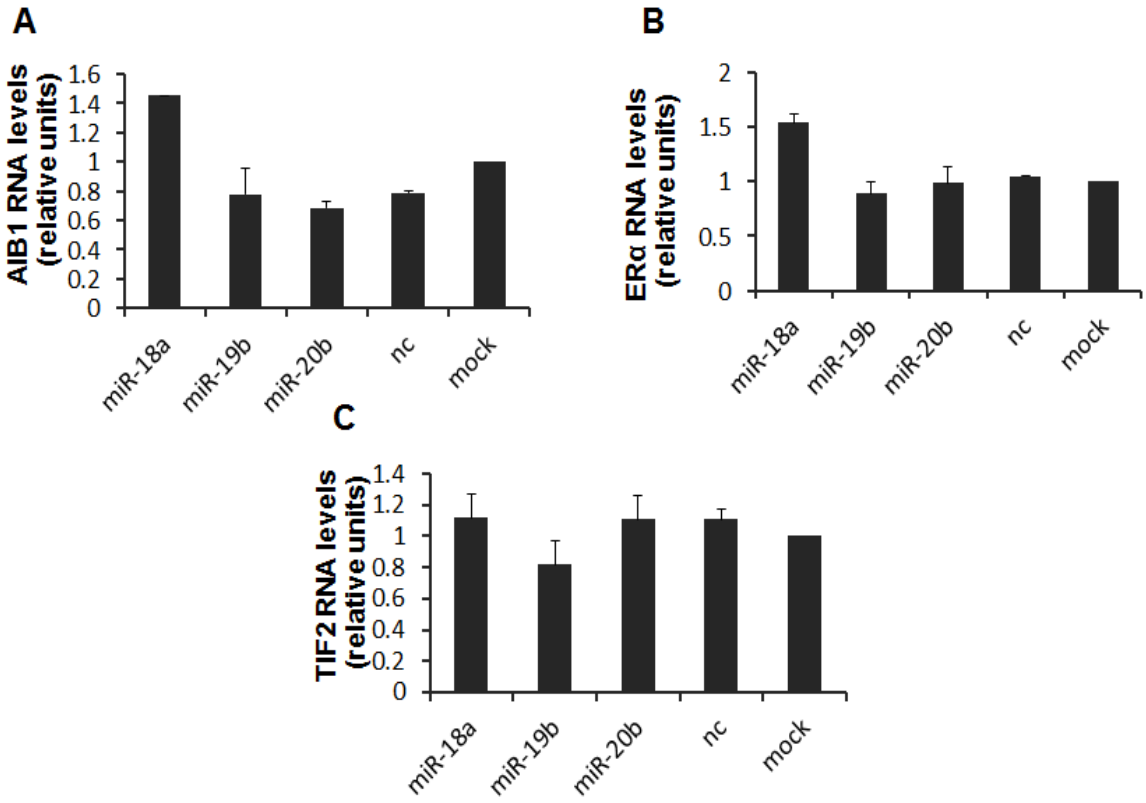


Figure 26: ERα and AIB1 mRNA levels do not change upon pre-miRNA overexpression. qRT-PCR analyses showing (A) AIB1, (B) ERα, and (C) TIF2 (negative control) in MCF-7 cells untransfected or transiently transfected with pre-miR-18a, pre-miR-19b, pre-miR-20b, and pre-miR-n.c for 48h. The mean of three independent experiments are presented, error bars represent s.e.m.

To check if these miRNAs had an effect on AIB1 after estradiol treatment, we transfected with 10nM of anti-miR-18a, anti-miR-20b, anti-miR-n.c. at different time points and measured the protein levels. AIB1 proteins levels were increased due to the inhibition of miR-20b over prolonged treatment (72h) of estradiol and no significant changes were found after inhibiting miR-18a and miR-n.c (Fig. 27A). Furthermore, transfection of anti-miR-20b in a dose dependent manner increased AIB1 protein levels (Fig. 27B).

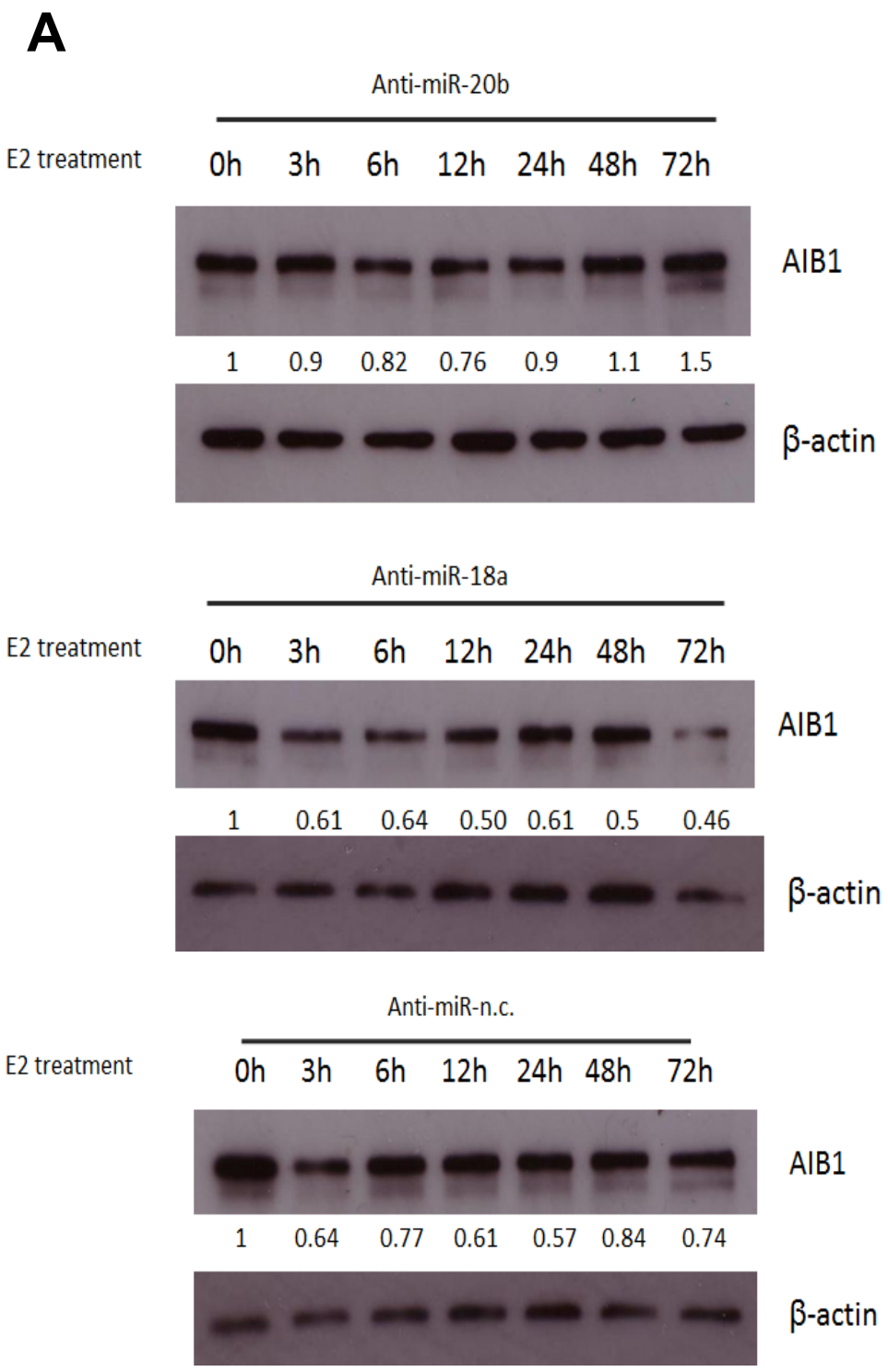


Figure 27: Inhibition of miR-20b leads to increase in AIB1 protein levels. (A) Western Blot showing the time point analysis of AIB1 and β-Actin in MCF7 cells transfected with anti-miR-20b, anti-miR-18a and anti-miR-n.c. at 10nM concentration. β-Actin was used as the loading control. One representative experiment from three independent experiments is shown. β-Actin was used as the loading control.

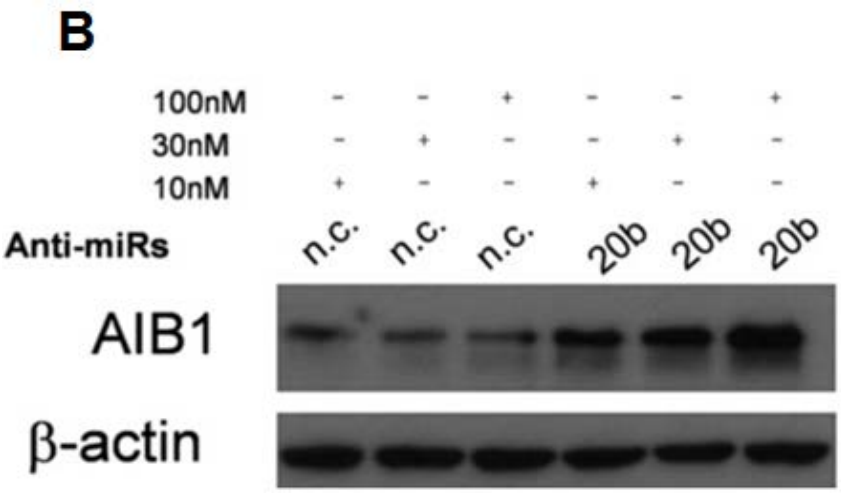


Figure 27: Inhibition of miR-20b leads to increase in AIB1 protein levels in a dose dependent manner. (B) Western blots showing AIB1 and β-actin in MCF-7 cells transfected with anti-miR-n.c and anti-miR-20b at 10, 30 and 100 nM concentrations. β-Actin was used as the loading control. One representative experiment from two independent experiments is shown.

Next we wanted to address whether these miRNAs negatively regulate ER by interacting with the predicted seed regions on its 3'UTR. To this end, we inserted into the luciferase reporter vector 4 fragments of the 3'UTR of ERα: the full length (FL containing all the putative miRNAs interaction sites); the first half of the 3'UTR (ERup for miR-17/20/106 and miR-18); a fragment containing just the putative miR-18 family interaction sites (ER1); and a fragment corresponding to the second half of the 3'UTR, containing the miR-19 family interaction sites (ER2) (Fig. 28A).

Transfection of miR-18a, miR-19b and miR-20b, derived from pri-mir-17-92, were used to investigate direct interactions with the 3'UTR of ERα constructs and we demonstrated that these miRNAs profoundly down-regulate luciferase activity for the constructs containing miRNA interaction sites, but not for the ones in which these sites are absent (Fig. 28B). This indicates direct targeting of ERα by a number of miRNAs derived from these paralogous primary miRNAs. We did not observe any down-regulation of luciferase reporter activity upon miR-17-5p over expression, according to a recent report [315].

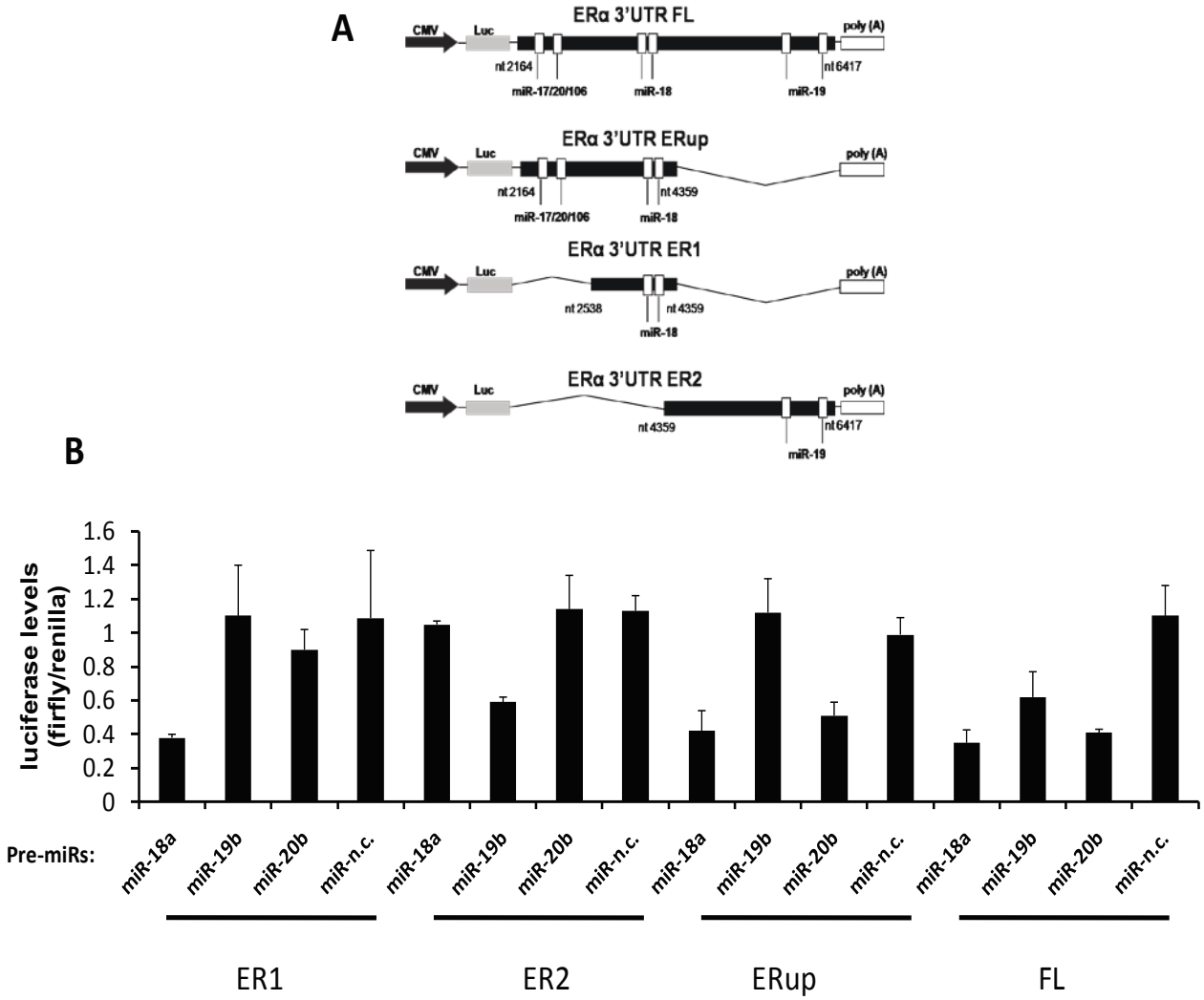


Figure 28: ERα is directly regulated by miRNA-3'UTR interaction. (**A**) Representation of the four different lengths of ERα 3'UTR cloned in the pMIR-REPORT luciferase vector and miRNAs interaction sites. (**B**) Luciferase activity from cells co-transfected with pre-miR-18a, pre-miR-19b, pre-miR-20b, pre-miR-n.c and different lengths of DNA fragments corresponding to the ERα 3'UTR. Firefly luciferase was normalized for transfection levels to Renilla luciferase as indicated in experimental procedures. The mean of three independent experiments are presented, error bars represent s.e.m.

Chapter-2 Results

3.2 Chapter - 2

3.2.1 Hypothesis

We have established that ER induces the transcriptional expression of mir-17-92 after E2 stimulation and this primary transcript is then processed by DROSHA in the nucleus, but the Dicer processing of the precursors derived from it is delayed over-time. Hence we hypothesized that E2 inducible factor may interact with the miRNA precursors derived from the mir-17-92 temporarily inhibiting their processing from Dicer in the cytoplasm. With this in mind, we aimed to establish the identity of the estrogen-inducible factors that could eventually cause this inhibition of miRNA processing. We used pre-mir-18a as a representative because it exerts the greatest effect on the ERα protein translational inhibition (Fig. 26). On the other hand, it is also possible that ER inducible factors interact with these precursors within the nucleus, retaining them inside this compartment, and avoiding exportation to the cytoplasm where Dicer can process them to mature miRNAs (Fig. 29). In summary these are our hypotheses:

Hypothesis 1: The microprocessor complex processes the primary transcript of the miR-17-92 following estrogen induced transcription, but these pre-miRNAs then remain in the nucleus and are not transported to the cytoplasm, preventing further processing by Dicer.

Hypothesis 2: The miR-17-92 derived pre-miRNAs are processed by the microprocessor complex and are actively transported out of the nucleus into the

cytoplasm where an inhibitor, interacting with the pre-miRNAs, inhibits further Dicer processing.

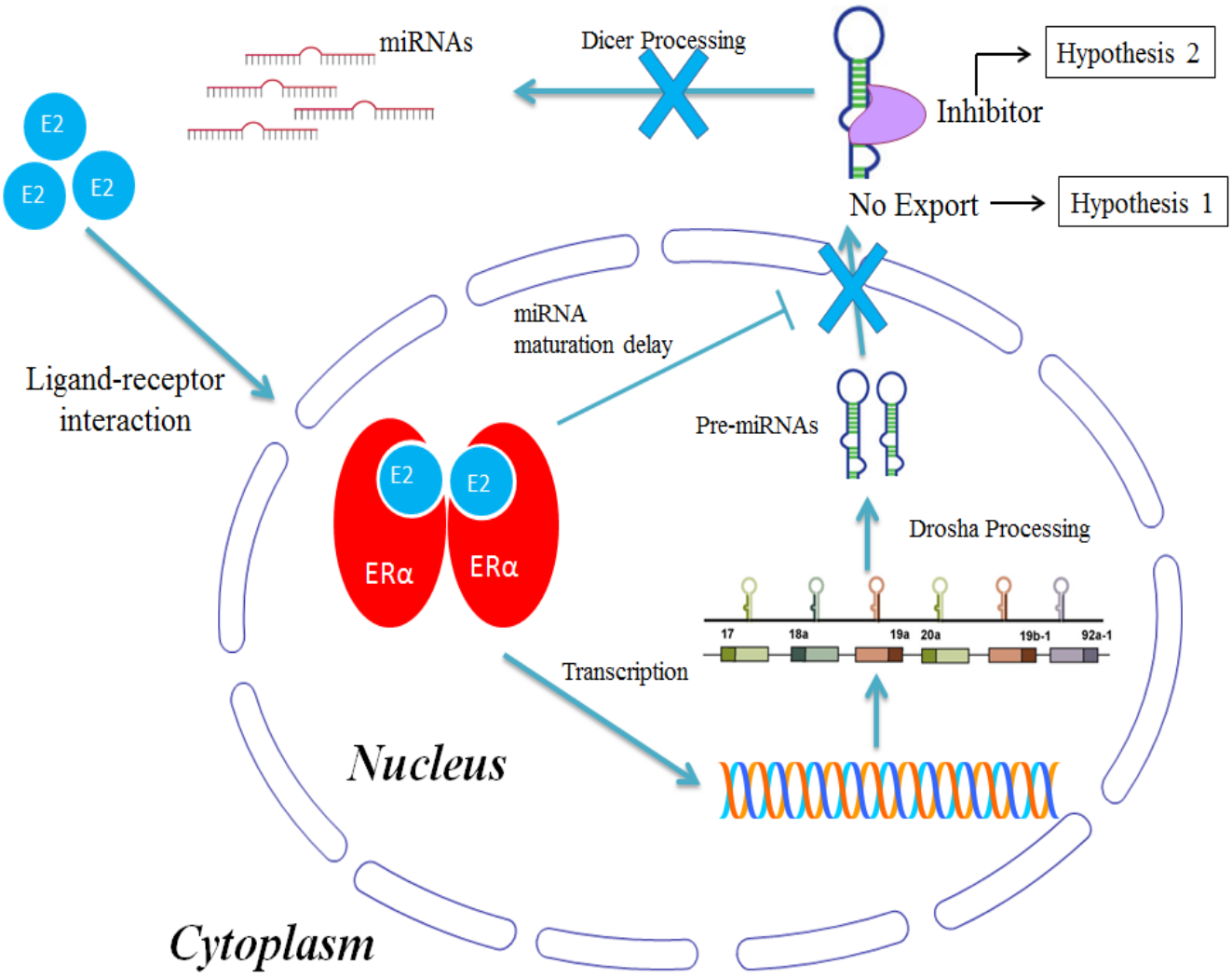


Figure 29: Diagramatic representation of the proposed hypothesis. We showed that after estrogenic activation, ERα upregulates the transcription of mir-17-92 via c-MYC but observed an inhibition at the processing of precursor to mature miRNAs Levels, implicating post-transcriptional regulation. We propose two possibilities: precursor molecules derived are retained into the nucleus thereby no Dicer processing (Hypothesis 1) or precursors is been transported from the nucleus to the cytoplasm where an inhibitor binds to it and inhibits Dicer processing (Hypothesis 2).

3.2.2 pre-miR-18a is been transported to cytoplasm

To check whether the precursors were retained in the nucleus after transcriptional upregulation of the cluster by estrogen receptor, we performed cytoplasmic RNA extraction and total RNA extraction (which includes nuclear and cytoplasmic extracts) after estradiol treatment.

We first treated MCF-7 cells with estradiol at timepoints 3h and 48h and then half of the cells were subjected to cytoplasmic extraction and half to total RNA extraction. We then went on to measure pre-miR-18a levels by RT-qPCR and found that the levels of pre-miR-18a was exactly the same when compared between cytoplasmic and total RNA extracts (Fig. 30A & B). There was an upregulation of pre-miR-18a levels after 3h of estradiol induction (2.5 fold) and subsequent reduction at 48h. This suggests that the pre-miR-18a molecule is transported into the cytoplasm, but Dicer mediated processing does not occur because an inhibitory factor interacts with the precursor (hypothesis 2).

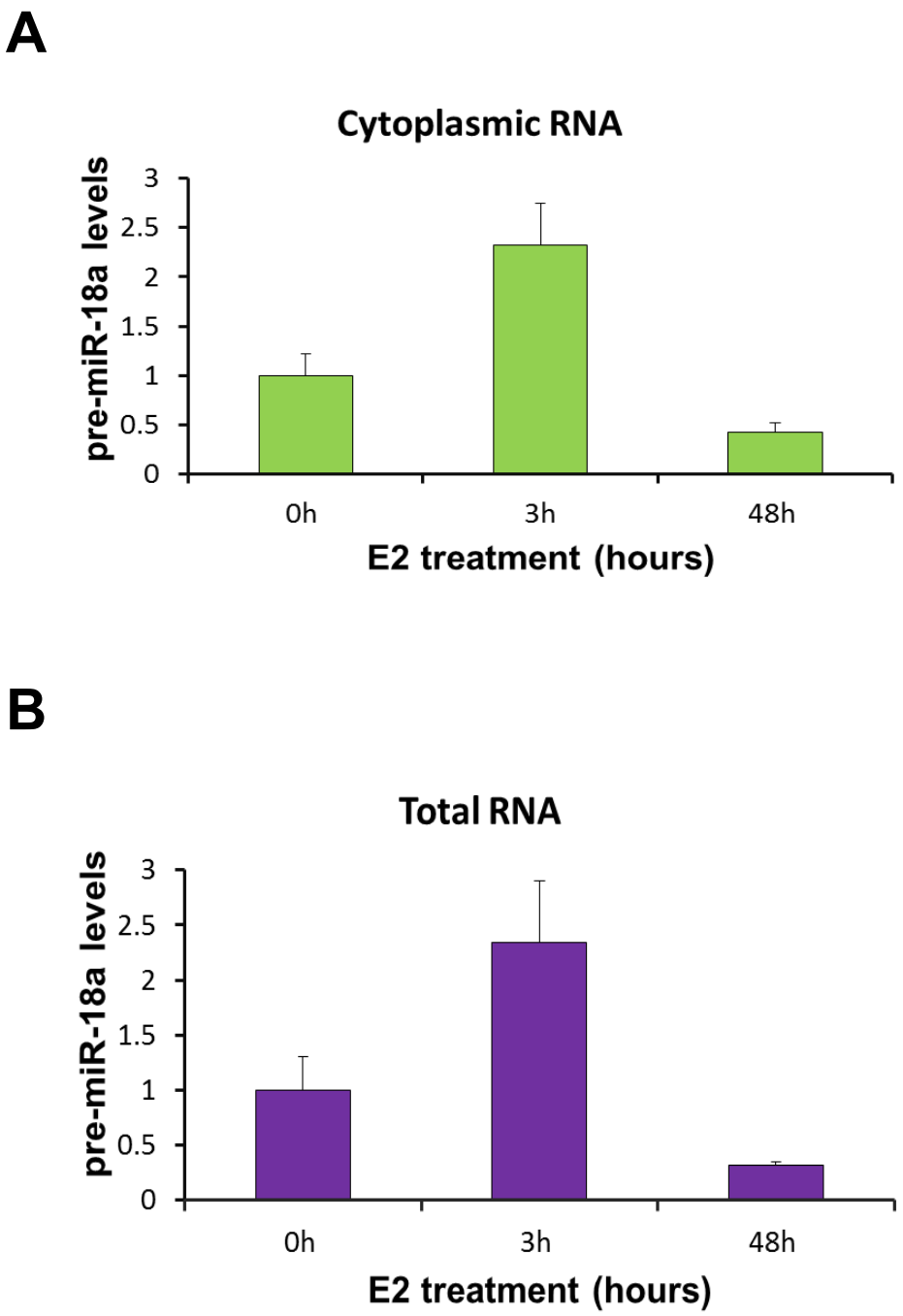


Figure 30: Cytoplasmic and Total RNA extracts after early (3h) and late (48h) estradiol induction. MCF-7 cells were maintained in DMEM (minus phenol red) supplemented with 10 % charcoal-dextran FCS for 3 days and then were either left untreated or treated with 10 nM E2 for 3h and 48h. **(A)** After cytoplasmic RNA extraction, expression of pre-mir-18a was analysed by RT-qPCR using SYBR green and normalized to GAPDH. **(B)** After total RNA extraction, expression of pre-mir-18a was analysed by RT-qPCR using SYBR green and normalized to GAPDH. The mean of three independent experiments are presented, error bars represent s.e.m.

3.2.3 Inhibitory molecule of mir-17-92 processing could be a protein shuttling between nucleus and cytoplasm

We transfected one set of MCF-7 cells with CMV promoter driven plasmid expressing mir-17-92 that expresses the primary transcript containing all the miRNAs derived from it and the other set with pre-miR-18a synthetic precursor. We then measured the levels of both the intermediates pri-mir-17-92 and pre-miR-18a and in addition the mature miR-18a molecules, by RT-qPCR. Although pri-mir-17-92 is processed to pre-miR-18a with high efficiency (Fig. 31A & B), pre-miR-18a to mir-18a processing was inhibited (Fig. 31C) when compared to the mock transfection, further validating our hypothesis that pre-mir-18a Dicer processing is inhibited in these breast cancer cells. Moreover, synthetic pre-miR-18a transfected alone was completely processed to miR-18a (Fig. 32A & B) when compared pre-N.C. This indicates that the factor(s) that inhibit the mir-17-92 processing are probably recruited either during transcription or during DROSHA processing in the nucleus and the formed miRNP complex gets transported to the cytoplasm. In this compartment the interacting inhibitor probably impedes the action of DICER on the pre-miRNA. A molecule that shuttles between the two main cellular compartments could be involved in this process.

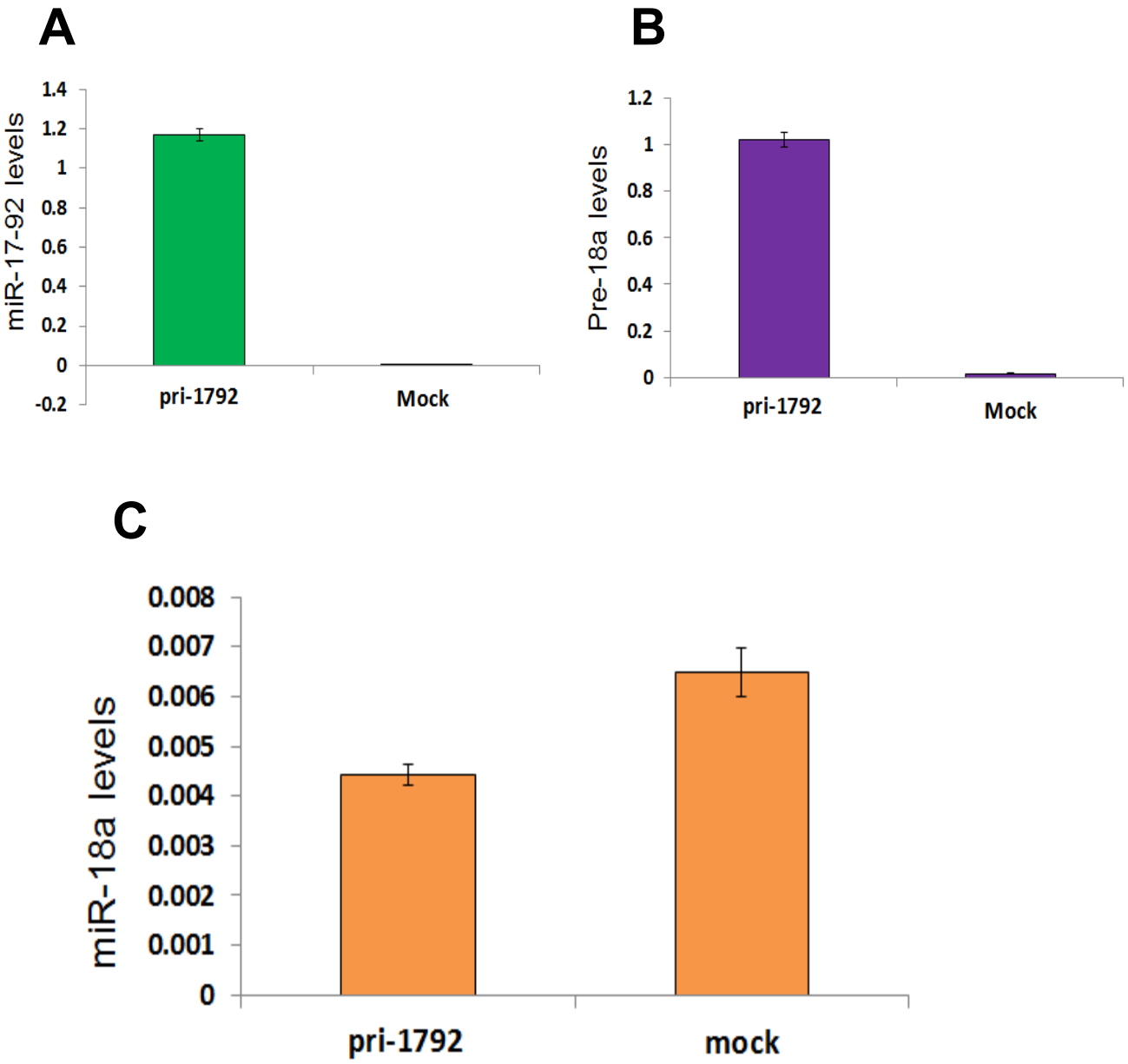


Figure 31: Overexpression of miR-17-92 cluster confirms the Dicer processing block of the cluster. MCF-7 cells were transfected with 1µg of pcmv-mir-17-92 and empty pcmv vector (mock) for 48h and then subjected to total RNA extraction. **(A)** Expression of mir-17-92 was analysed by RT-qPCR using SYBR green and normalized to U6 snRNA **(B)** Expression of pre-miR-18a was analysed by RT-qPCR using SYBR green and normalized to U6 snRNA. **(C)** Expression of miR-18a was analysed by RT-qPCR using Taqman assay and normalized to U6 snRNA. The mean of three independent experiments are presented, error bars represent s.e.m.

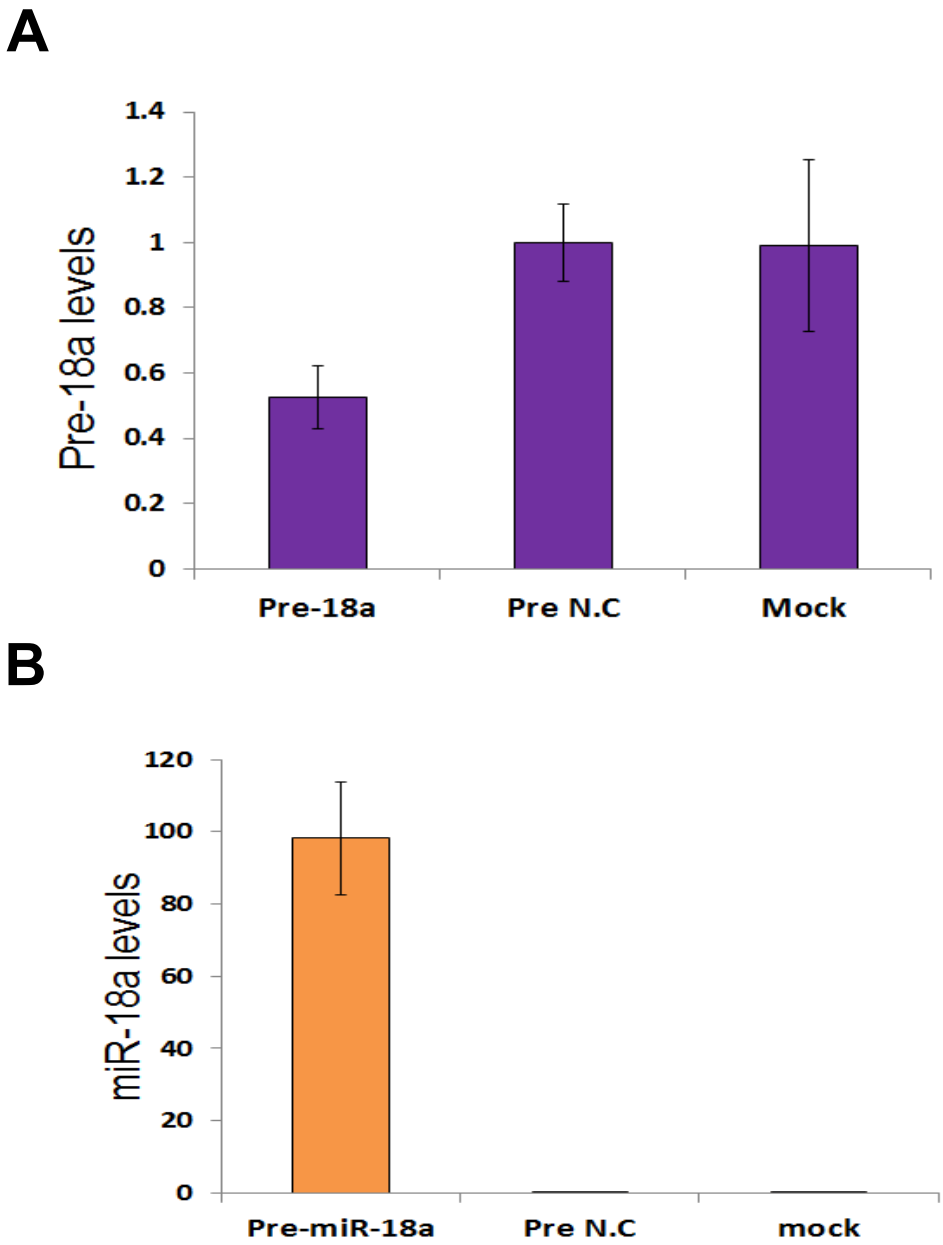


Figure 32: Overexpression of synthetic pre-miR-18a shows pre-miR-18a is completely processed to miR-18a. MCF-7 cells were transfected with 5nM of pre-miR-18a and pre-N.C for 48h; also untreated (mock) was included and then subjected to total RNA extraction. **(A)** Expression of pre-miR-18a was analysed by RT-qPCR using SYBR green and normalized to U6 snRNA **(B)** Expression of miR-18a was analysed by RT-qPCR using Taqman assay and normalized to U6 snRNA The mean of three independent experiments are presented, error bars represent s.e.m.

3.2.4 Preliminary investigation of RNA binding proteins by Mass spectrometry

To identify the miRNA regulating factor we took advantage of a mass spectrometry approach. The complexity and dynamic nature of the proteome presents massive technological challenges. Mass spectrometry is a powerful analytical technology that enables interpretation of protein sequences. Over recent years improved mass spectrometry approaches like increased sensitivity and higher resolving power have been developed to characterize the proteome, indicating it as powerful approach for identification of factors that could inhibit miRNA biogenesis[316]. In addition, mass spectrometry based proteomics has been previously employed to identify several critical components of the miRNA biogenesis pathway and their post-translational modifications, amongst which is LIN-28 [317-319].

In order to discover such a molecule using this approach, we performed an affinity chromatography approach followed by mass spectrometry, using miRNA precursor molecules joined with beads as bait. We used pre-miR-18a as the specific molecule, and pre-miR-34a, as negative control. This approach allowed us to obtain a list of proteins containing RNA binding domains that specifically interact with pre-miR-18a alone, pre-miR-34a alone or both molecules, and in response to E2 treatment.

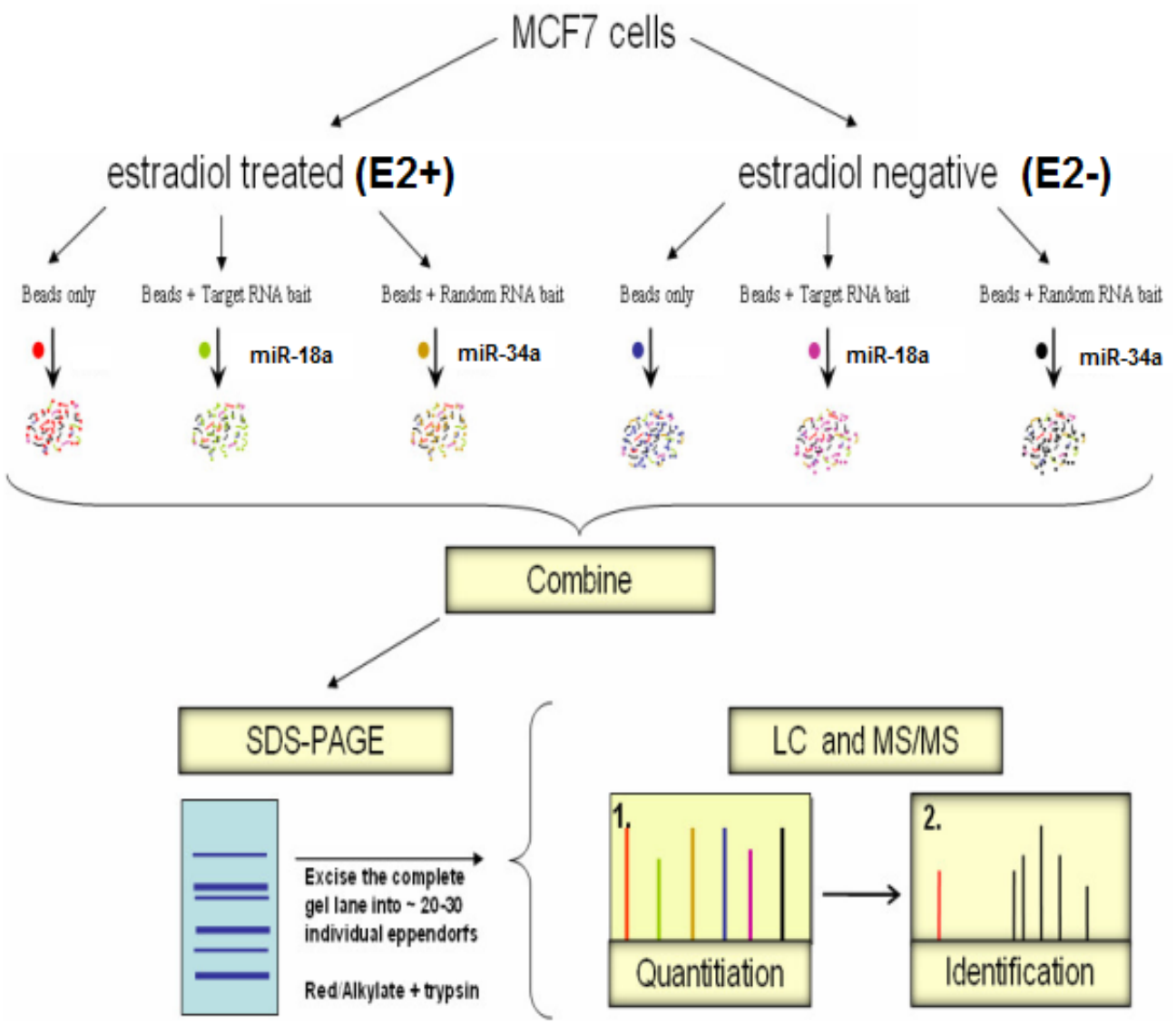


Figure 33: A schematic to illustrate the overall outline of the mass spectrometry experiment. MCF-7 cells were maintained in DMEM (minus phenol red) supplemented with 10 % charcoal-dextran FCS for 3 days and then were either left untreated or treated with 10 nM E2 for 6h. Cells were then subjected to protein extraction and subsequent treatment with agarose beads bound with the pre-miRs accordingly (shown above). Proteins were separated on 4-12% gradient gels before purification of the protein bands and mass spectrometry for their identification (Figure 34).

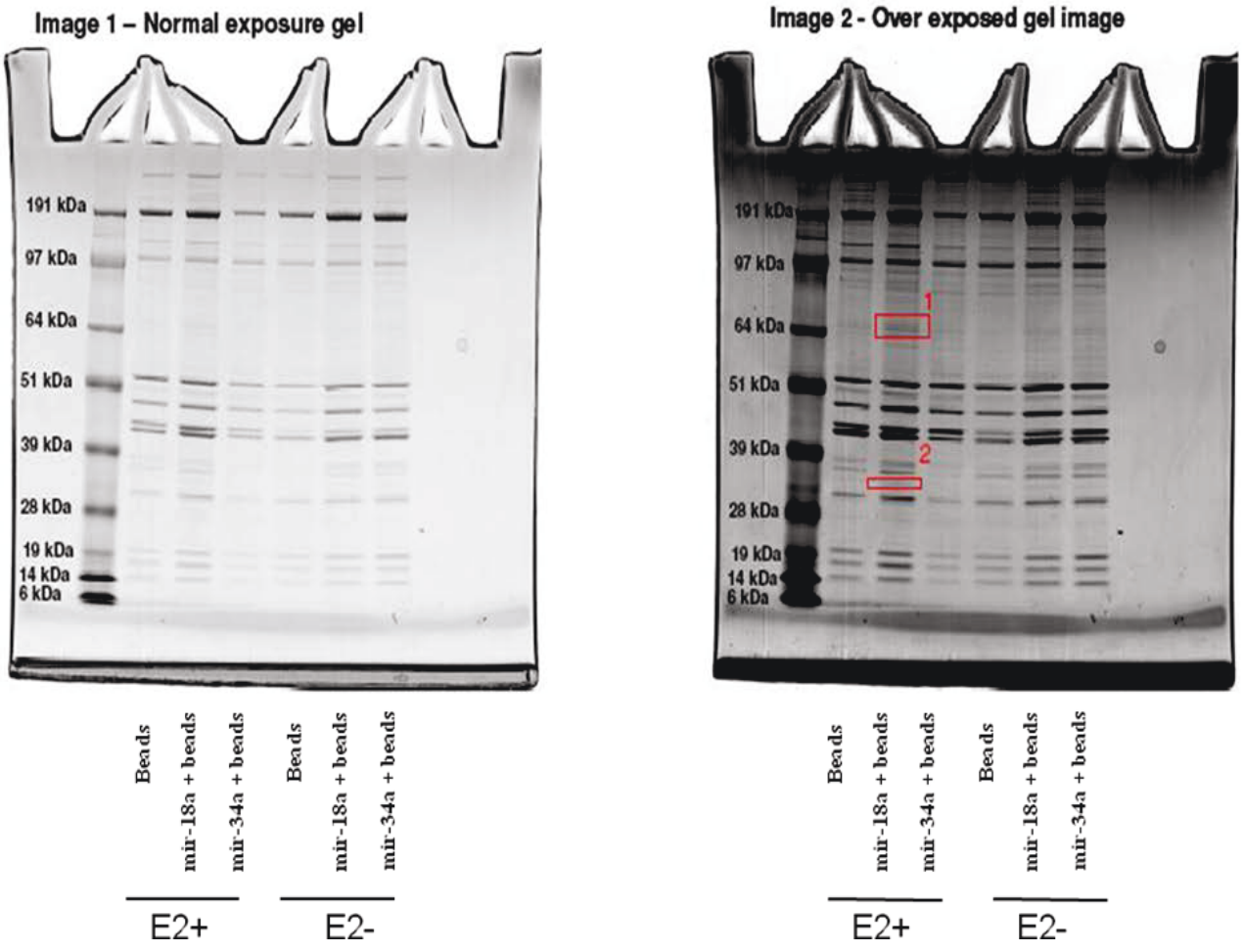


Figure 34: Image of the gels, normal exposure and over exposed image. In both the image (from left to right) samples are SeeBlue Plus Molecular weight markers, beads only (E2+), beads + pre-miR-18a (E2+), beads + pre-miR-34a (E2+), beads only (E2-), beads + pre-miR-18a (E2-), beads + pre-miR-34a (E2-). Two gel cuts have been made from the unlabelled sample gel, as shown in the figure above. Proteins were digested and analysed by LC/MS/MS over a 30 minute gradient. LC/MS/MS raw data files were processed and search against the SwissProt database, using the Mascot search engine.

3.2.5 Idenfication and Screening of selected RBPs

We chose proteins based on the number of peptides bound to the beads. Proteins were identified by LC-MS were specifically chosen from estradiol treated pre-miR-18a and pre-miR-18a/ pre-miR-34a samples; and not in control samples are listed. Ribosomal proteins and cyto-skeletal proteins are excluded from this list.

Table 11: List of selected RBPs from Mass Spectrometry experiment

Pre-miR-18a (E2+)		**Pre-miR-18a/Pre-miR-34a (E2+)**	
Accession	**Description**	**Accession**	**Description**
TNRC6B _HUMAN	Trinucleotide Repeat-Containing Gene 6B Protein	**PC4_HUMAN**	Pheochromocytoma Cell-4
ZNF225 _HUMAN	C2-H2 Type Zinc Finger Protein	**TOE1_HUMAN**	Target Of EGR1 Protein 1
hnRNPA1 _HUMAN	Heterogeneous Nuclear Ribonucleoprotein A1	**ANKRD17_HUMAN**	Ankyrin Repeat Domain 17
THOC4/ALY _HUMAN	THO Complex Subunit 4	**ASB7_HUMAN**	Ankyrin Repeat And SOCS Box Containing 7
FGD1 _HUMAN	FYVE, RhoGEF And PH Domain Containing 1	**NONO_HUMAN**	Non-POU Domain Containing, Octamer-Binding
CTTN _HUMAN	Src substrate cortactin	**SF3B3_HUMAN**	Splicing Factor 3b,

			Subunit 3, 130kDa
ZNF168 _HUMAN	Zinc Finger Protein 168	**STK19_HUMAN**	Serine/Threonine Kinase 19
DAZAP1 _HUMAN	DAZ Associated Protein 1	**U2AF1_HUMAN**	U2 Small Nuclear RNA Auxiliary Factor 1
EDC4 _HUMAN	Human Enhancer Of Decapping Large Subunit	**RPL35_HUMAN**	Ribosomal Protein L35
CPSF6 _HUMAN	Cleavage And Polyadenylation Specific Factor 6	**METTL3_HUMAN**	Methyltransferase Like 3
STAT3 _HUMAN	Signal Transducer And Activator Of Transcription 3	**RBM34_HUMAN**	RNA Binding Motif Protein 34
HSPA1A _HUMAN	Heat Shock 70kDa Protein 1A	**PCF11_HUMAN**	Polyadenylation and cleavage Factor Subunit
HSPA8 _HUMAN	Heat Shock 70kDa Protein 8		
HSPB1 _HUMAN	Heat Shock 27kDa Protein 1		

Interestingly, we found a few important heat shock proteins in the list (particularly in E2 treated pre-miR-18a beads samples only) so we went on to investigate any involvement of heat shock proteins in the miRNA biogenesis of mir-17-92 family or even global biogenesis. We did not identify any change of

miR-18a, miR-19a, miR-20a and miR-34a levels after silencing of the three HSP proteins (HSPA1A, HSPA8 and HSPB1).

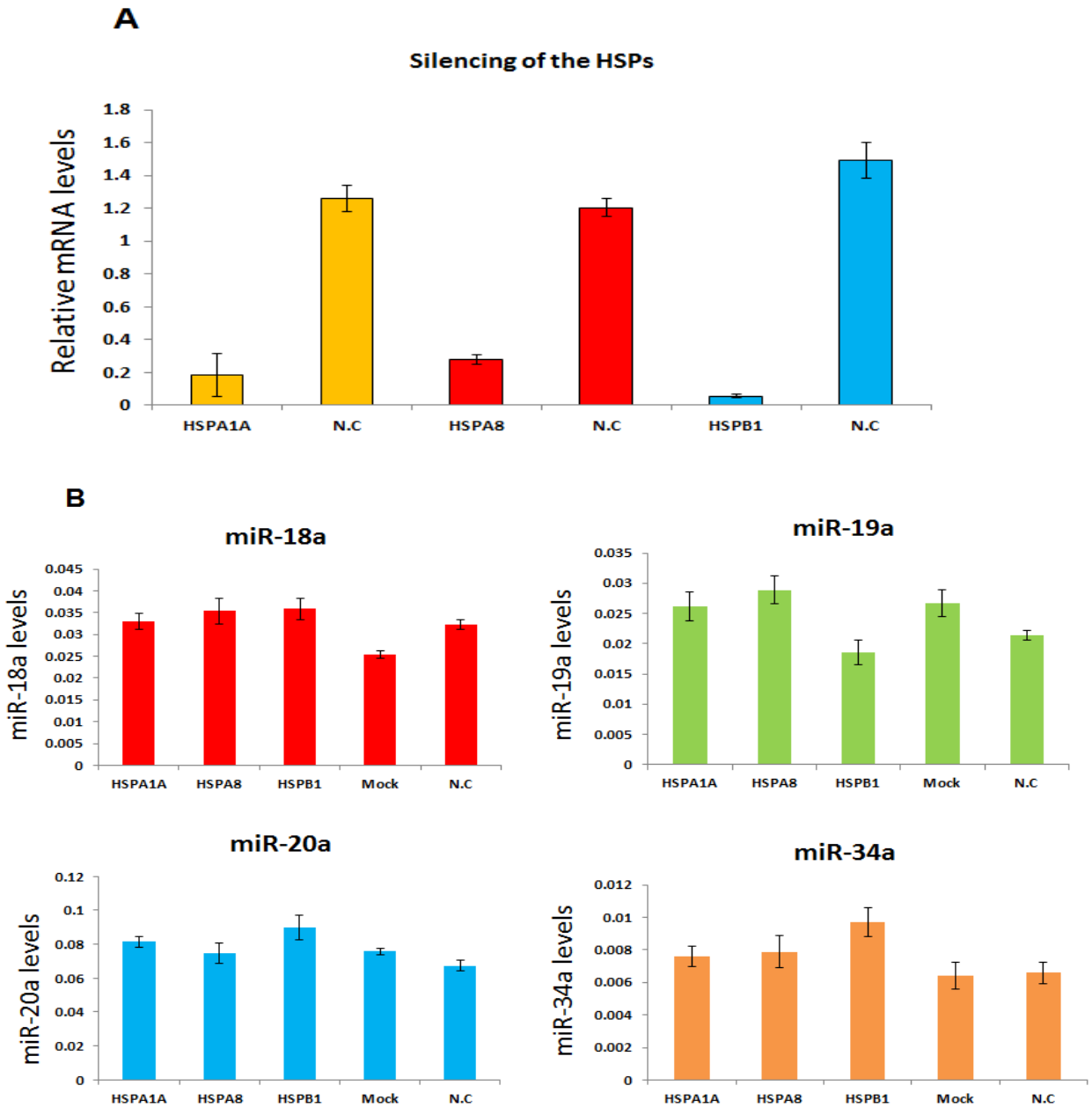


Figure 35: Silencing of HSP proteins (HSPA1A, HSPA8 and HSPB1) does not affect miRNA biogenesis. MCF-7 cells were transfected with 20nM of siRNAs targeting HSPA1A, HSPA8 and HSPB1 for 72h and then subjected to total RNA extraction. **(A)** Expression of mRNAs levels of HSPA1A, HSPA8 and HSPB1 were analysed by RT-qPCR using SYBR green and normalized to GAPDH **(B)** Expression of miR-18a, miR-19a, miR-20a and miR-34a were analysed by RT-qPCR using Taqman assay and normalized to U6 snRNA. One representative experiment from two independent experiments is shown in figure 35.

3.2.6 ALY, possible regulator of miRNA biogenesis?

We subsequently performed siRNA based screening for other possible RBPs that could be involved in the mir-17-92 biogenesis. We screened selected RBPs based on the number of peptides bound to pre-miR-18a and pre-miR-18a/ pre-miR-34a to check whether it is involved in either mir-17-92 or global processing. The following RBPs were chosen: ALY, ANKRD17, DAZAP1, SF3B3, CTTN, hnRNPA1. We did not find any significant changes after silencing these RBPs (Fig. 36A). We did observe a slight decrease in miR-18a, miR-21 and miR-34a after silencing of ALY, indicating a possible involvement in global processing. We were finding it extremely difficult to get consistent results using RT-qPCR (which is further discussed in chapter 4.2). Hence we performed northern blotting, a recognised and superior technique for quantification of miRNA levels. Expression of miR-18a and miR-34a is low in MCF7 cells so levels were undetectable, unlike miR-21, which is quite highly expressed in most cancer cells. We found that silencing of ALY decreased miR-21 levels when compared to the negative control (Fig. 36B); hence raising the possibility of its involvement as a positive regulator of miRNA processing.

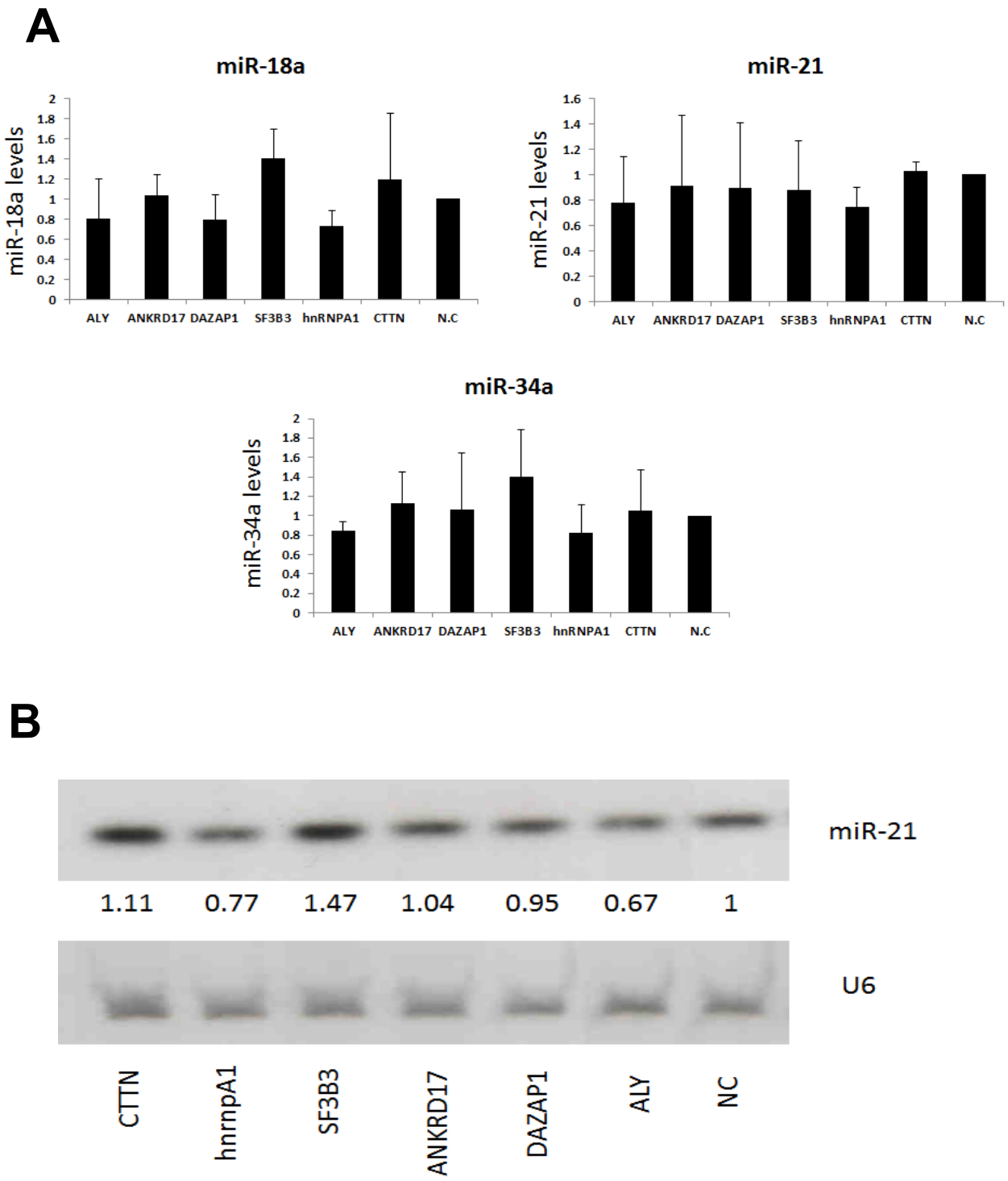


Figure 36: Screening of selected RBPs using RT-qPCR and northern blotting. MCF-7 cells were transfected with 20nM of siRNAs targeting ALY, ANKRD17, DAZAP1, SF3B3, CTTN, hnRNPA1 and N.C (negative control siRNA) for 72h and then subjected to total RNA extraction. **(A)** miRNA levels of miR-18a, miR-21 and miR-34a was analysed by RT-qPCR using Taqman assay and normalized to U6 snRNA. The mean of six independent experiments are presented, error bars represent s.e.m. **(B)** Northern blot analysis (30µg of total RNA was used) of miR-21 after silencing ALY, ANKRD17, DAZAP1, SF3B3, CTTN, hnRNPA1 and N.C for 72h and normalized to U6 snRNA. One representative experiment from two independent experiments is shown.

In our previous chapter (chapter 3.1) we showed an inhibitory effect of Dicer processing on all miRNAs that arise from the mir-17-92 transcript, but here silencing of ALY decreased levels of miR-18a, miR-21 and miR-34a (Fig. 36A&B). This raises the possibility that ALY could be involved in global miRNA biogenesis.

We also wanted to ensure that the effect observed was not due to transcriptional regulation but instead, regulation at the post-transcriptional level, indicative of processing regulation. Therefore, in the same samples, we quantified the levels of two other miRNA biogenesis products (primary transcript and precursor) in addition to the miRNA, using RT-qPCR. Using the primary transcript and precursor of miR-18a and miR-21, we showed that silencing of ALY only altered miRNA levels but not primary transcript or precursor levels, revealing a possible regulation at the Dicer step (Fig. 37 A&B).

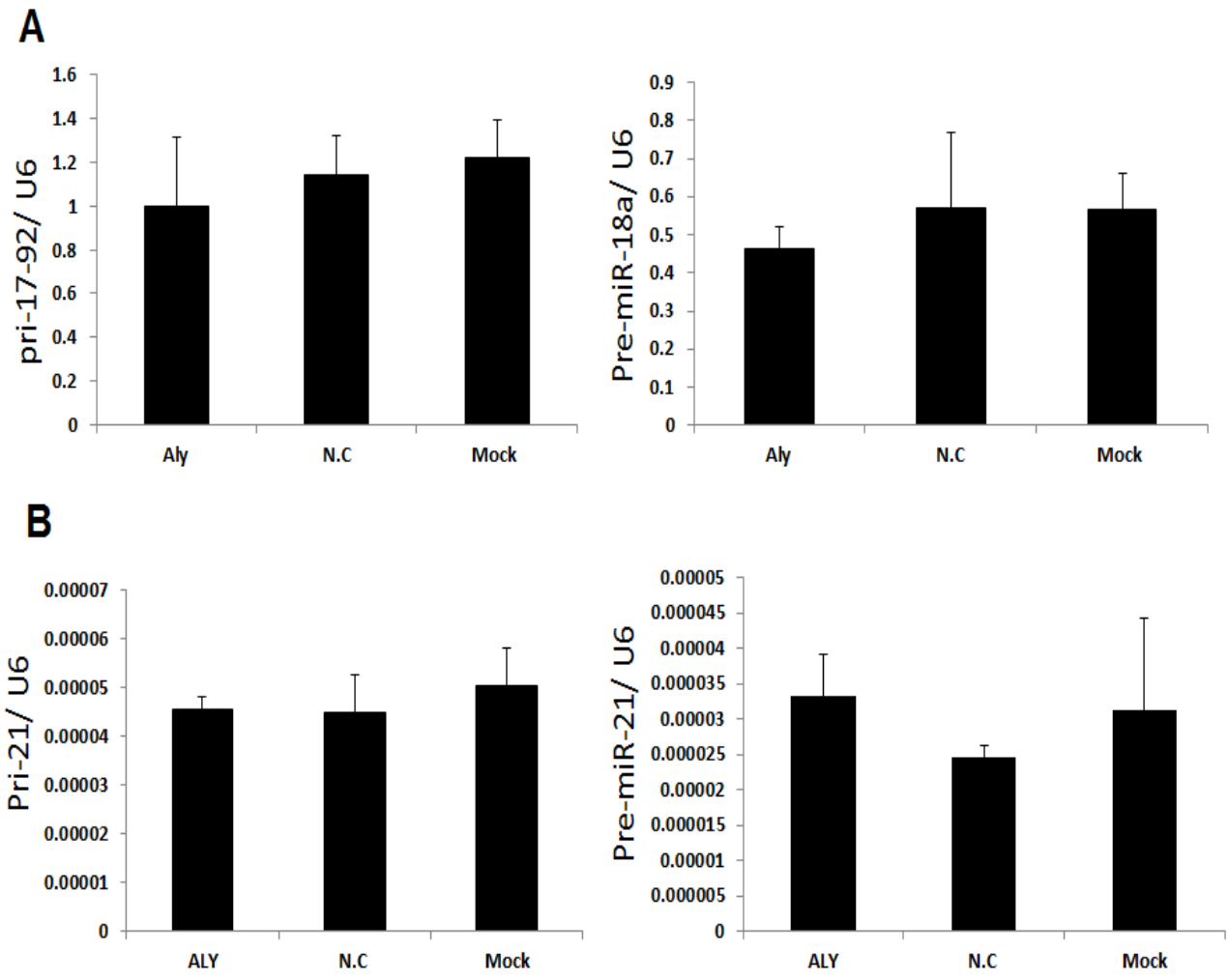


Figure 37: primary transcript and precursors levels did not change after silencing of ALY. MCF-7 cells were transfected with 20nM of siRNAs targeting ALY for 72h and then subjected to total RNA extraction. **(A)** Primary transcript and precursor levels of miR-18a were analysed by RT-qPCR using SYBR green and normalized to U6 snRNA. **(B)** Primary transcript and precursor levels of miR-21 were analysed by RT-qPCR using SYBR green and normalized to U6 snRNA. The mean of two independent experiments are presented, error bars represent s.e.m.

Interestingly, ALY seems to be one of the key factors involved in the nuclear transport of mRNA (Fig. 37) and it is a protein known to shuttle between the nucleus and cytoplasm [320-322]. It acts as a molecular adaptor for key proteins like UAP56, TAP/NXF1 involved in the mRNA transport [323].

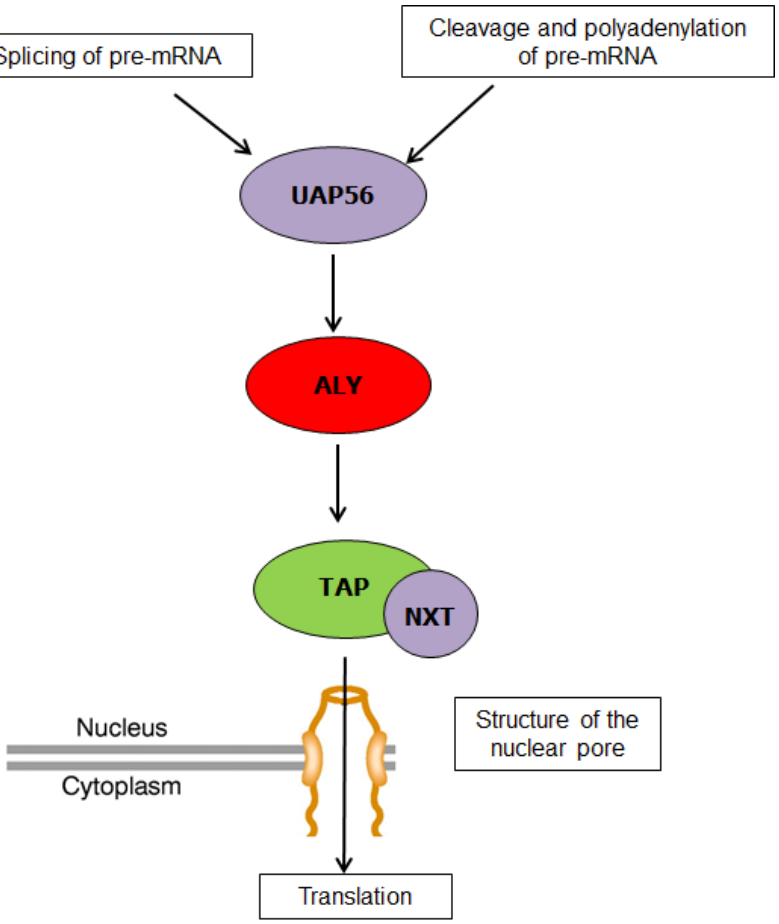


Figure 38: Key factors involved in the mRNA transport. ALY acts as a molecular adaptor for the important proteins involved in mRNA transport. Modified from [324].

We checked the ALY mRNA and proteins levels after its silencing to make sure that we obtained a good silencing level (Fig. 39 A&B).

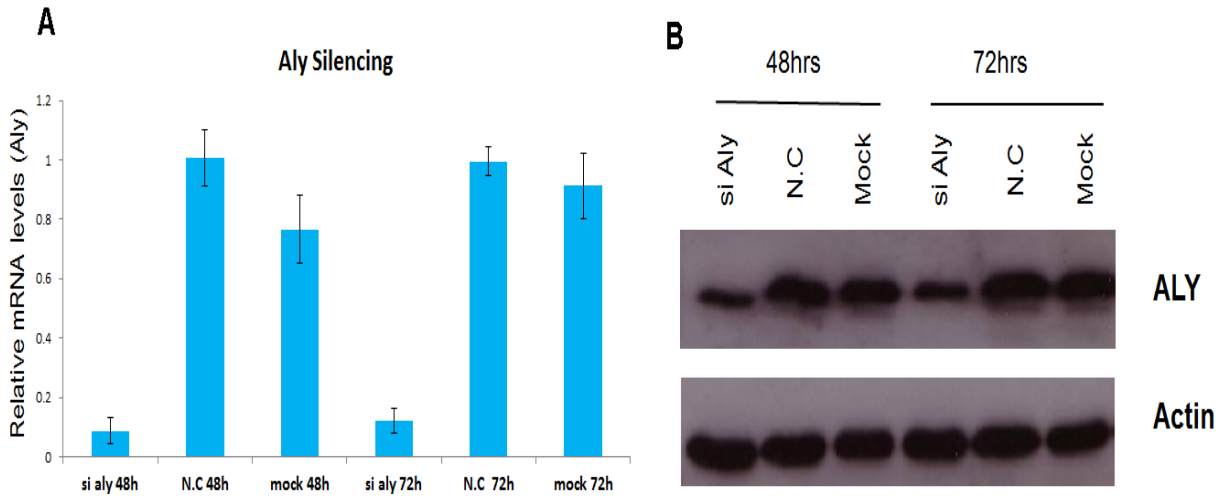


Figure 39: mRNA and protein levels of ALY after silencing using siRNA. MCF-7 cells were transfected with 20nM of siRNAs targeting ALY, N.C and mock (untransfected) for 48h and 72h respectively; then subjected to total RNA and protein extraction. **(A)** mRNA levels of ALY after silencing were analysed by RT-qPCR using SYBR green and normalized to GAPDH. The mean of two independent experiments are presented, error bars represent s.e.m. **(B)** Western blot analysis of ALY after silencing for 48h and 72h; β-Actin was used as the loading control. One representative experiment from two independent experiments is shown.

We next wanted to investigate whether ALY interacts directly with the miRNA precursors or indirectly through other RBPs. We performed electrophoretic mobility shift assay (EMSA) using recombinant ALY protein and radiolabeled γ-^{32}P pre-miR-18a to show that the formation of this RNA-protein complex was specific. We did see that ALY formed a complex together with pre-miR-18a and did not form any complex in the absence of pre-miR-18a, indicating that the interaction is specific (Fig. 40A). We further went on to investigate the potential sites of the precursor for ALY interaction. We performed EMSA again with entire precursor (pre-miR-18a), terminal loop (TL) of the precursor, double strands of the precursor, miRNA strand of the precursor (miR-18a) and complementary stand of the precursor (miR-18a*). We found that ALY requires the entire precursor molecule (pre-miR-18a) to interact, there was no complex formed with any other parts of the precursor molecule (Fig. 40B).

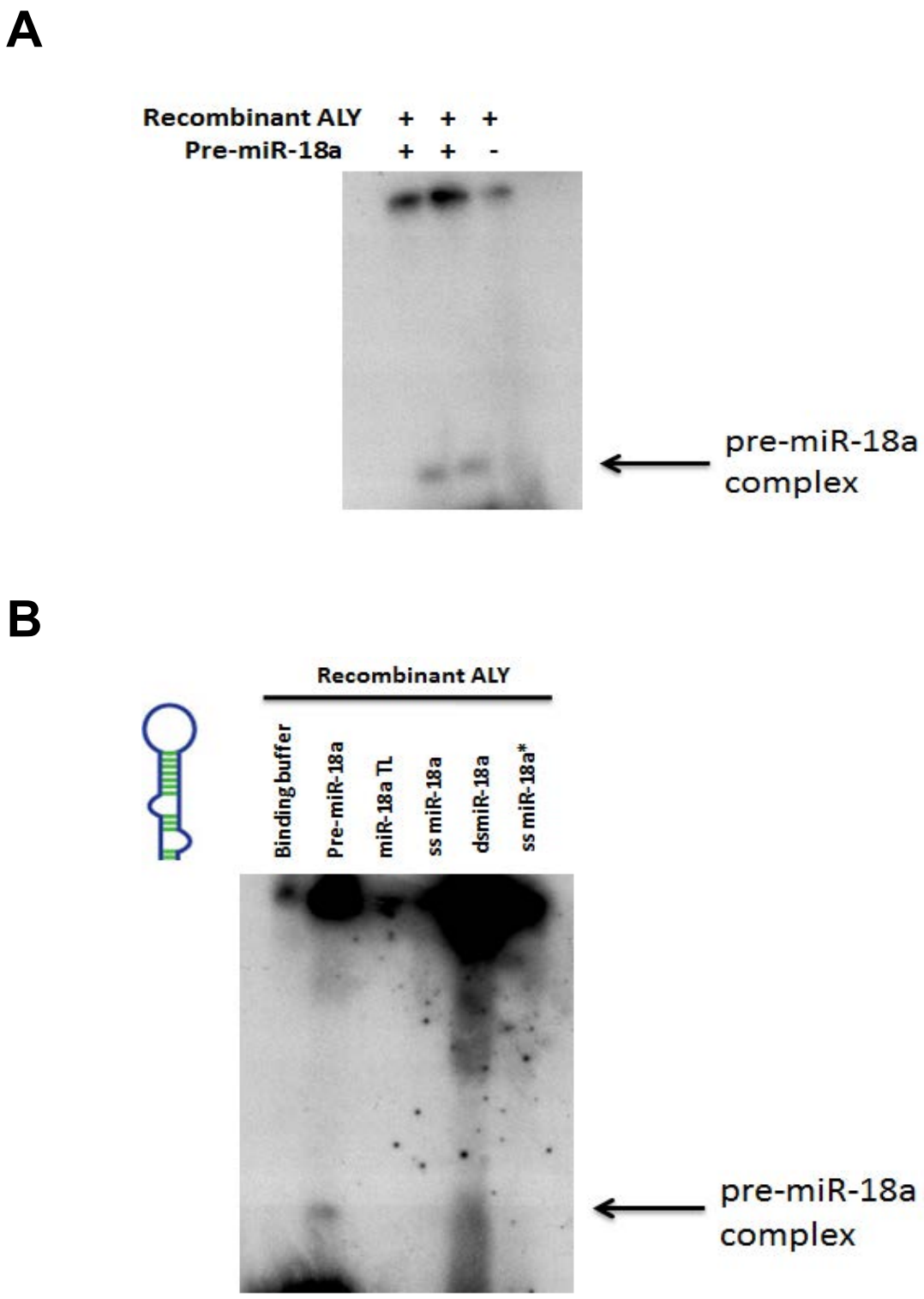


Figure 40: EMSA revealing interaction of ALY with the pre-miR-18a. EMSA performed using γ-^{32}P labelled pre-miR-18a and recombinant ALY. Reactions were subjected to electrophoresis on 12% polyacrylamide gels containing 5% glycerol in TBE (0.5X). Protein: RNA complexes were detected by nondenaturing gel electrophoresis and autoradiography. **(A)** Specific binding of pre-miR-18a by ALY **(B)** ALY binds to entire pre-miR-18a not any other specific part of the precursor molecule.

We went on to perform *in vitro* pre-miRNA processing assay to enable us characterize the role of ALY in the processing of pre-miRNAs to mature miRNAs but unfortunately we were unable to identify any role of ALY involved in the processing (data not shown). Overexpression of ALY with a plasmid also failed to show any change in the mature miRNA levels or the precursor levels. Furthermore, we performed RNA immunoprecipitation to see the interaction of ALY with pri-, pre- and mature miR-18a but again did not find an interaction between any of the biogenic products (data not shown). We were finding it extremely difficult to have consistent results and I have discussed these problems in chapter 4.2.

We carried screening for other RBPs which may be involved in the processing. Further screens using RT-qPCR and northern blotting to assess levels of miRNAs after silencing of RBPs identified one potentially interesting candidate, RBM34 . RBM34 is an RNA binding protein of unknown function. We also used two different siRNA controls to minimize the false positive results.

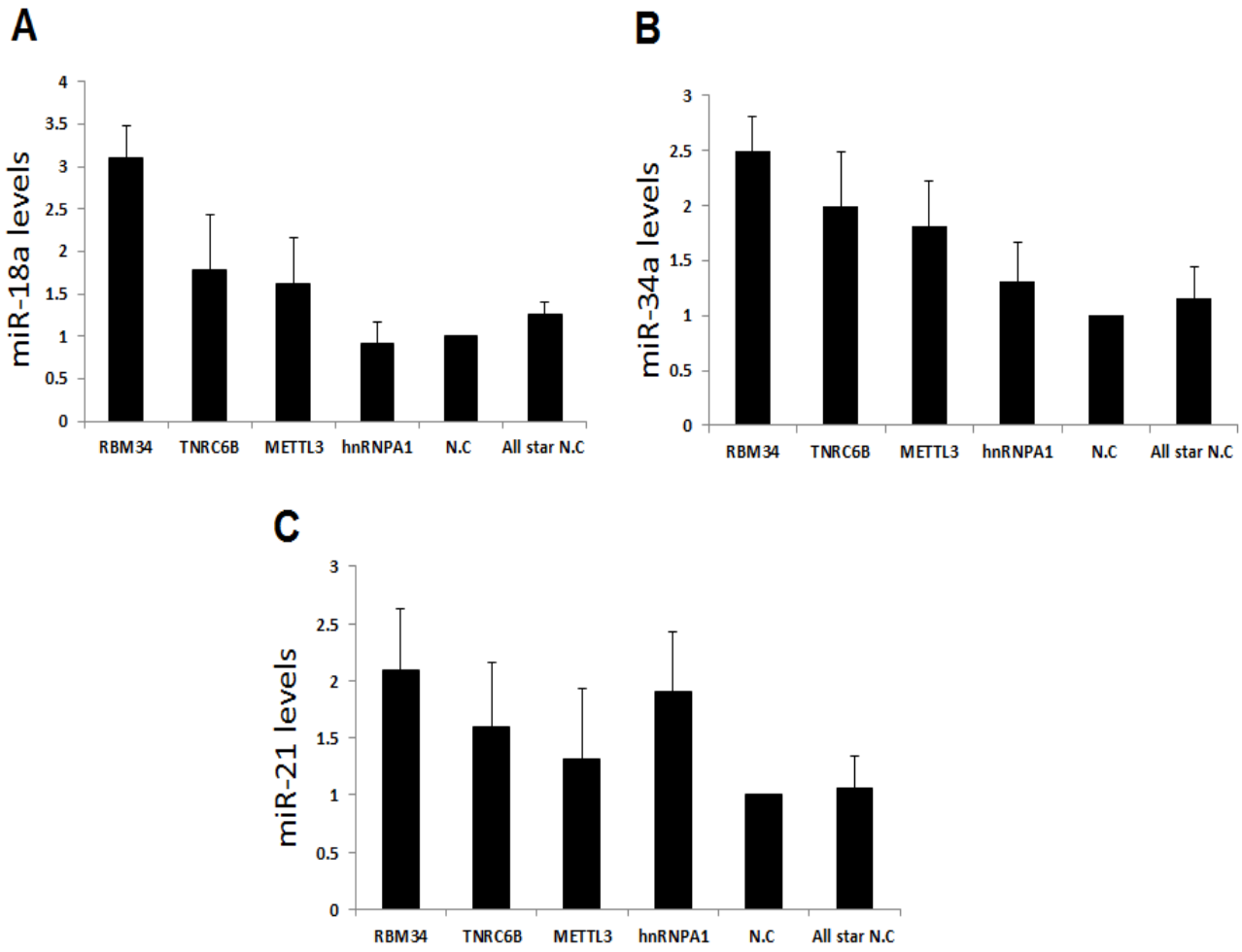


Figure 41: Screening of selected RBPs using RT-qPCR. MCF-7 cells were transfected with 20nM of siRNAs targeting RBM34, TNRC6B, METTL3, hnRNPA1, N.C and all star N.C for 72h and then subjected to total RNA extraction. miRNA levels of **(A)** miR-18a **(B)** miR-34a **(C)** miR-21 were analysed by RT-qPCR using Taqman assay and normalized to U6 snRNA. The mean of three independent experiments are presented, error bars represent s.e.m.

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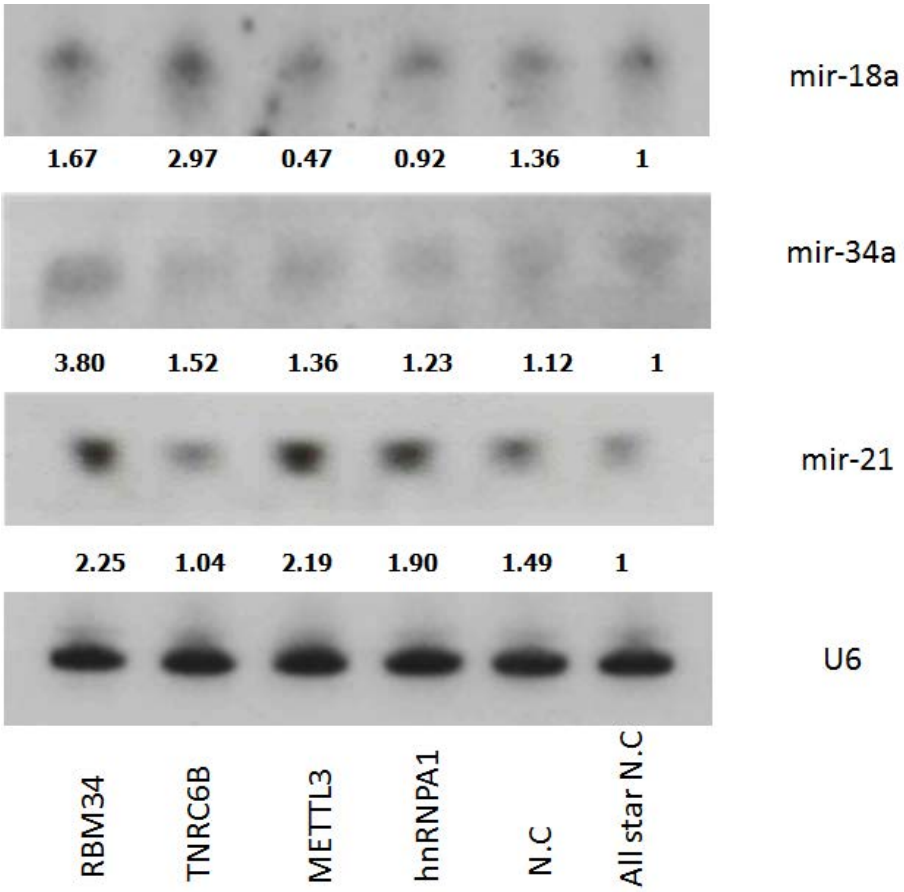

Figure 41: Screening of selected RBPs using RT-qPCR and northern blotting. (D) Northern blot analysis (50µg of total RNA was used) of miR-18a, miR-21 and miR-34a after silencing RBM34, TNRC6B, METTL3, hnRNPA1, N.C and all star N.C for 72h and normalized to U6 snRNA. One representative experiment from two independent experiments is shown.

RBM34 silencing increased mature levels of both miR-18a and miR-34a (Fig. 42A), without effect on precursors levels (Fig. 42B). This indicates that RBM34 is able to reduce the expression of both miRNAs at DICER processing step. In our previous chapter (chapter 3.1) we showed an inhibitory effect of Dicer processing relating to all miRNAs that arise from the mir-17-92 transcript, and we appreciated here an effect of RBM34 on both miR-18a and miR-34a (Fig. 42A). Reviewing the mass spectrometry data, this protein was present in both the pull-down with the beads (pre-miR-18a and pre-miR-34a). Although we cannot rule out the possibility that the inhibitory mechanism that we previously demonstrated is acting also on miR-34a, further experiments will demonstrate if the inhibitory action of RBM34 on these miRNAs is related to the estrogenic response.

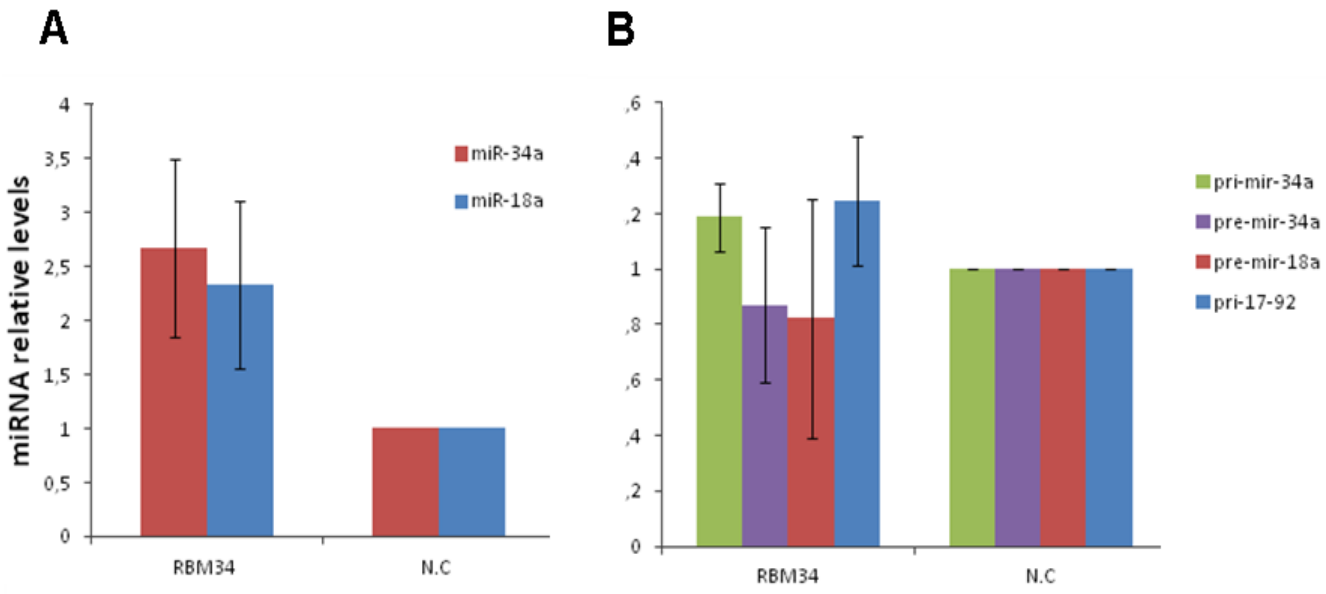

Figure 42: RBM34 silencing increases miR-34a and miR-18a levels. MCF-7 cells were transfected with 20nM of siRNAs targeting RBM34 for 72h and then subjected to total RNA extraction. Expression levels of **(A)** miR-34a and miR-18a were analysed by RT-qPCR using Taqman assay and normalized to U6 snRNA. **(B)** pri-17-92, pri-34a, pre-miR-18a and pre-miR-34a by RT-qPCR using SYBR green and normalized to U6 snRNA. The mean of three independent experiments are presented, error bars represent s.e.m.

We carried out further experiments with overexpression of RBM34 plasmid and again we did not find any change in the mature miRNA levels or the precursor levels. We also performed RNA immunoprecipitation to see the interaction of RBM34 with pri-, pre- and mature miR-18a but again unfortunately we couldn't find interaction between any of the biogenic products (data not shown).

We decided not to carry on with the project as we were finding it extremely difficult with the inconsistent results and technical difficulties we had during this time, I have discussed these problems later in chapter 4.2.

Chapter-3 Results

3.3 Chapter-3

3.3.1 Introduction

As previously mentioned, most breast tumours express ERα [325] and patients with ERα positive disease respond to anti-estrogens (tamoxifen), estrogen withdrawal (aromatase inhibitors) and direct targeting of the receptor (fulvestrant) [29]. The introduction of these treatments has had a profound impact on patient survival [326]. However, resistance to these therapies is common [327]. Since phosphorylation is one of the most important post-translational modifications most of the proteins undergo and has critical roles in the regulation of many cellular processes. *In vitro* evidence points to the importance of ERα phosphorylation [328] in the development of endocrine resistance [329, 330]. RNA interference (RNAi) screens in drug discovery are a commonly used technique, particularly in the search for anti-cancer drugs.

Hence, kinomes have become a target for large scale genomics, enabling the systematic screening for a role for individual kinases in regulating different proteins. We wanted to perform 'Kinome' screening using an siRNA library which targeted all kinases in the genome and identify a novel kinase regulator of ERα. Thus, our intention was to find a new novel kinase that regulates ERα that could possibly be involved in endocrine resistance mechanisms.

3.3.2 Kinome Screen set-up

The kinase library [Human Kinase Set V3.0 siRNA library (Qiagen)] used in the following study has siRNAs which target 691 kinases with each well having two pooled siRNAs targeting every individual kinase to have silencing efficacy. We decided to perform our screen in the most well characterised ER positive cell line, MCF-7. We further carried on preliminary experiments checking the transfection efficiency using transfection reagent, HiPerfect and also siRNA silencing efficacy by choosing 5 random kinases from the library FRK, FYN, HCK, BTK and CSF1R. We transfected the chosen siRNAs in 24-well format (which will be used for the screen) for 72h and was subjected to total RNA extraction using RNeasy Kit (Qiagen). We checked the mRNAs levels of the individual kinases using RT-qPCR and expression of all the five genes were supressed suggesting the efficacy of siRNAs from the library (Fig. A,B,C,D & E). We observed more than 80% of the gene silencing in all five genes transfected with respective siRNAs, when compared to the negative control (CT siRNA) indicating that the entire library can be safely used to perform the planned screening.

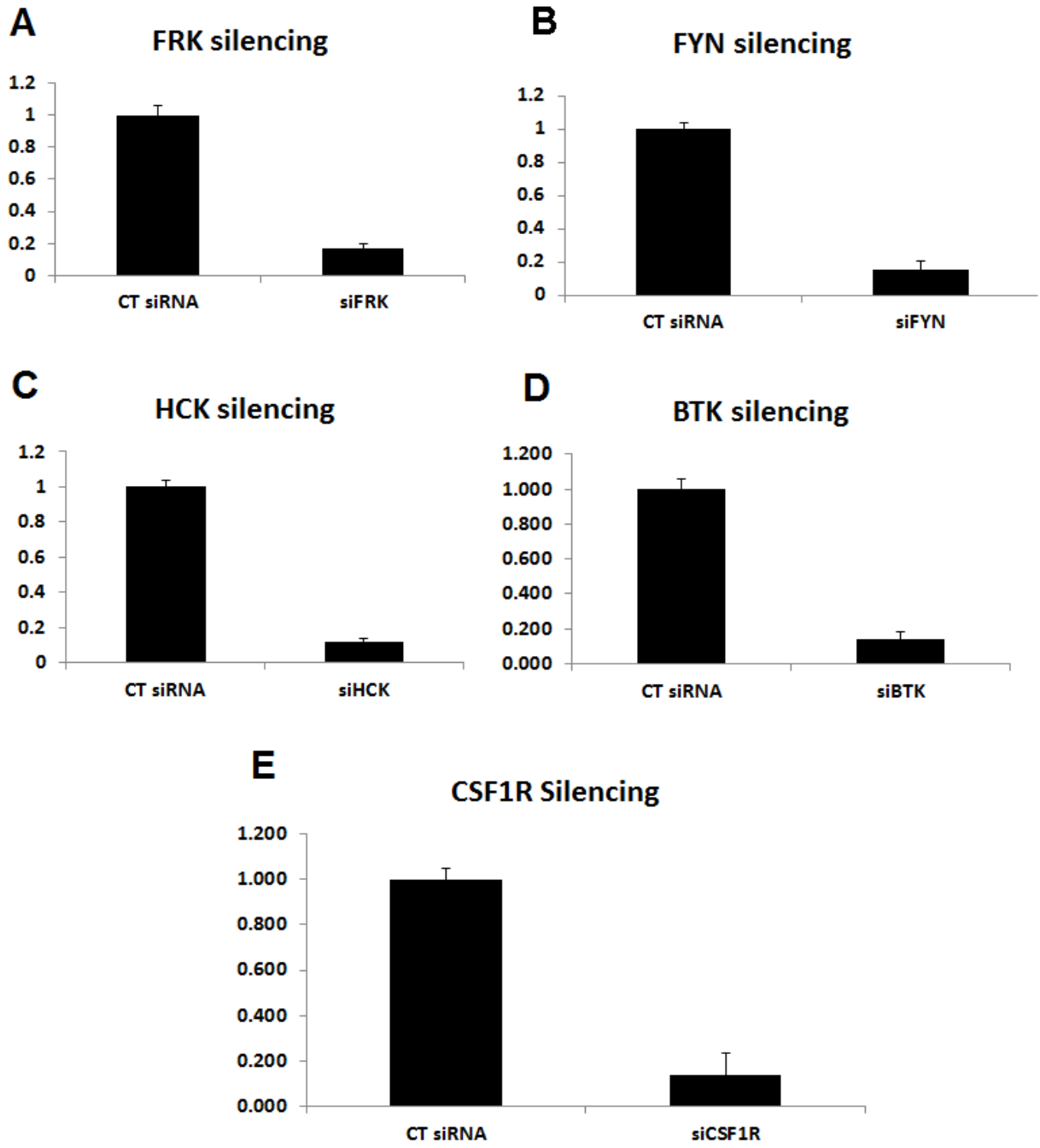

Figure 43: Silencing of the selected kinases using the siRNAs from the library. MCF-7 cells were transfected with 20nM of siRNAs targeting FRK, FYN, HCT, BTK and CSF1R for 72h respectively; then subjected to total RNA extraction. mRNA levels of **(A)** FRK **(B)** FYN **(C)** HCT **(D)** BTK **(E)** CSF1R after silencing was analysed by RT-qPCR using Taqman assay and normalized to *GAPDH*. Error bars represent standard deviation of 2 experiments each in triplicate.

To check estrogenic induction by estradiol (E2), we chose two genes well known to be upregulated by ERα transcriptional activity, TFF1 [331] and GREB1 [332]. We performed estradiol induction with the kinases we chose for the previous experiment (Fig. 43). We transfected MCF-7 with the chosen siRNAs and after 48h of transfection the cells were replaced with the media either treated with E2 or untreated and incubated for another 24h. We quantified the mRNAs levels of the TFF1 and GREB1 using RT-qPCR and expression of both genes was appropriately increased, confirming that the treatment was effective (Fig. 44 A&B). We then silenced the well-known kinases that regulate ERα activity, mitogen-activated protein kinase-3 (MAPK3) and AKT which phosphorylate ERα at Ser118 and Ser167 respectively [253, 330]. We transfected MCF-7 with MAPK3 and AKT siRNAs, after 48h of transfection the cells were replaced with the media either treated with E2 or untreated and incubated for another 24h. As expected, silencing of MAPK3 and AKT kinases led to reduction of TFF1 mRNA levels after estrogenic induction when compared to the mock and negative siRNA control, indicating that the screening can detect new kinases involved in estrogenic signalling (Fig. 44C). We decided to consider the estrogen-responsive gene TFF1 as readout after silencing of all the kinases.

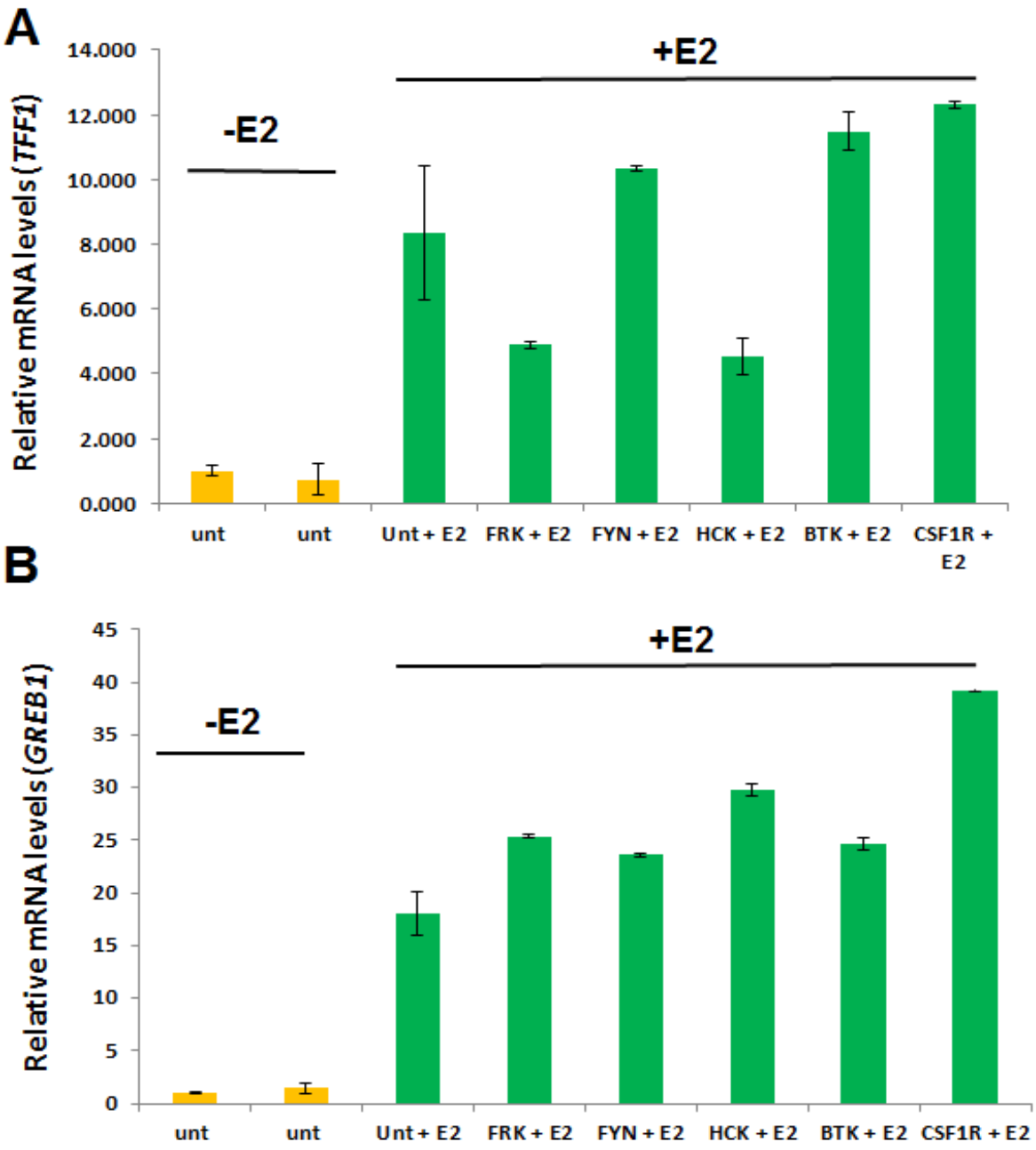

Figure 44: Estradiol induction and silencing of the selected kinases using the siRNAs from the library. MCF-7 cells were transfected with 20nM of siRNAs targeting FRK, FYN, HCT, BTK, CSF1R, MAPK3 and AKT, after 48h of transfection the cells were replaced with the media either treated with E2 or untreated, incubated for another 24h and then subjected to total RNA extraction. **(A)** mRNA levels of TFF1 was analysed by RT-qPCR using Taqman assay and normalized to GAPDH. **(B)** mRNA levels of GREB1 was analysed by RT-qPCR using Taqman assay and normalized to GAPDH. Error bars represent standard deviation of 2 experiments each in triplicate.

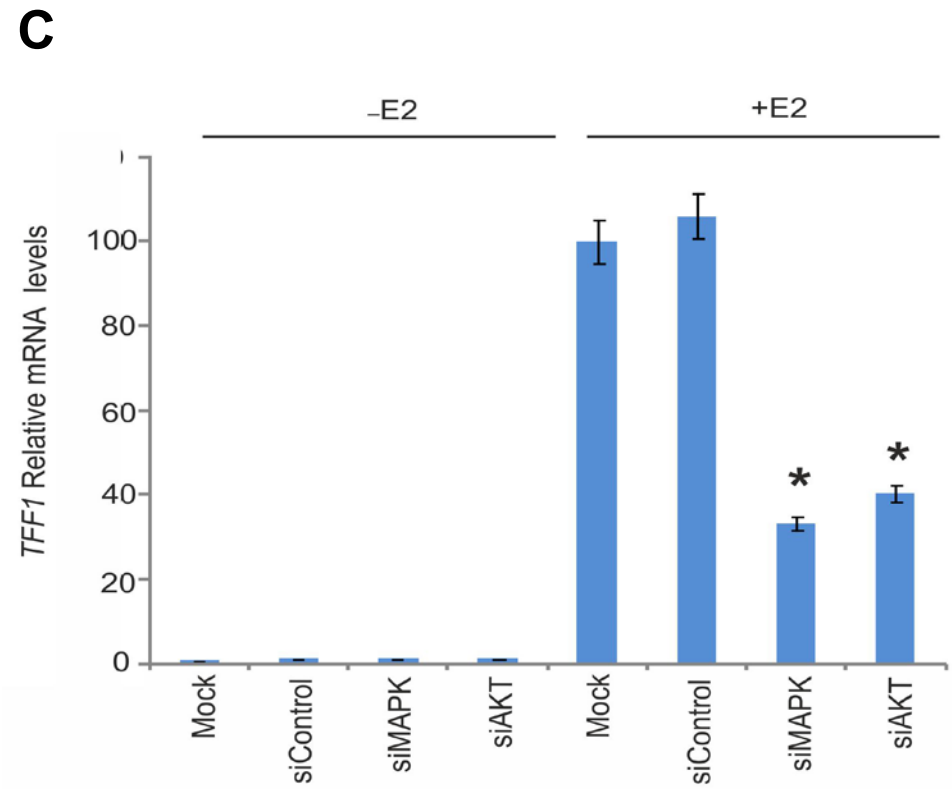

Figure 44: Estradiol induction and silencing of known kinases that regulate TFF1 expression using the siRNAs from the library. (C) Transfection of MCF7 cells with MAPK or AKT siRNAs followed by treatment with E2 significantly inhibited the expression levels of TFF1. Error bars represent standard deviation of 2 experiments each in triplicate [* $p < 0.05$ compared to siControl (Student's t test)].

3.3.3 Screening of the Kinome

To identify kinases that regulate ERα activity, we performed the siRNA screen using expression of the estrogen-responsive TFF1 gene as readout for altered ERα activity and also using appropriate controls provided in the library. The schematic representation below (Fig. 45) displays the way we performed the screening.

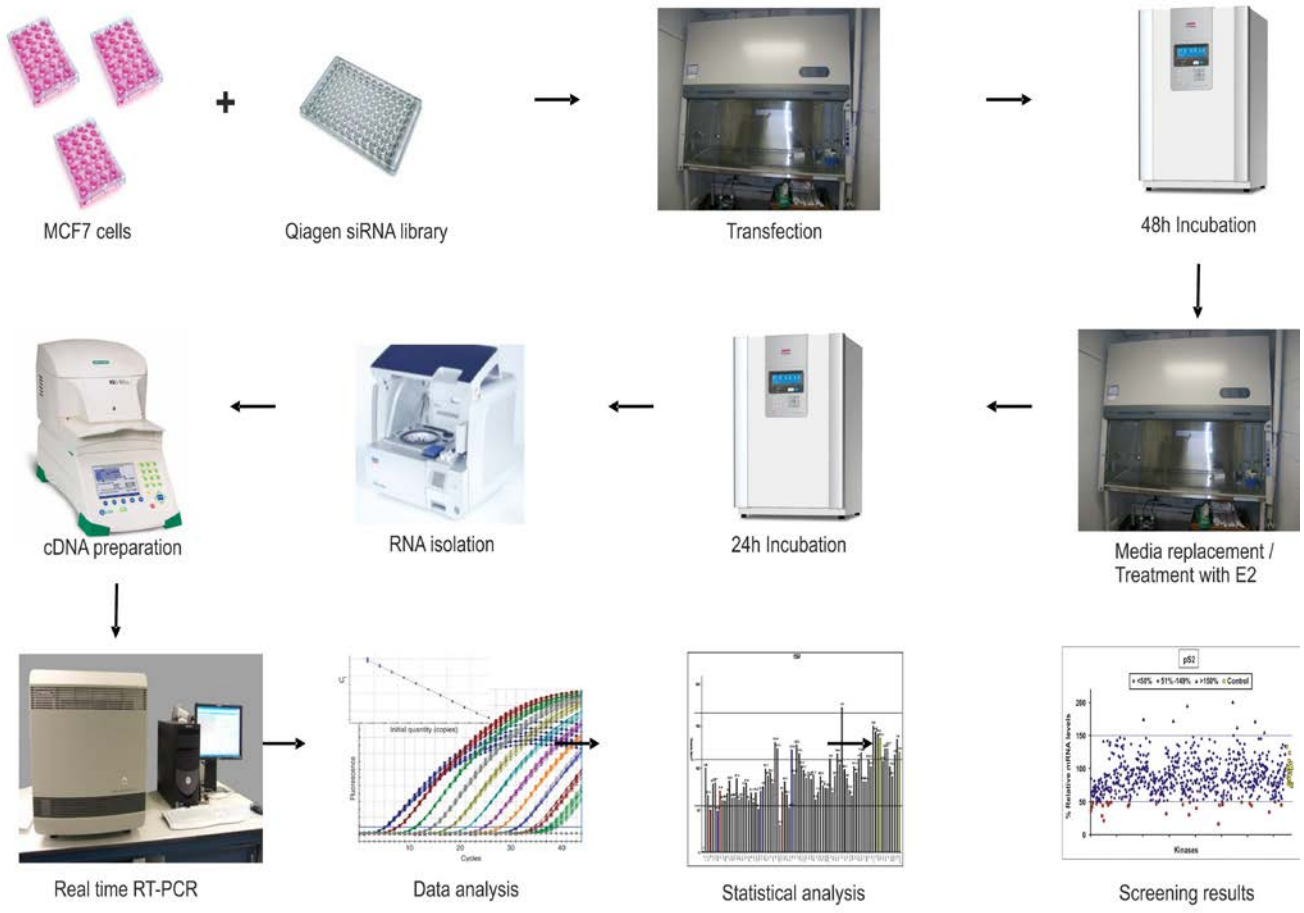

Figure 45: Schematic representation of the experimental procedure for the screening. MCF-7 cells were plated in a 24-well plates and transfected with 20nM of siRNAs targeting the entire kinome (691 kinases) and appropriate controls were used which was provided in the library. After 48h of transfection, the cells were replaced with the media treated with E2 or untreated (as control to check the estradiol induction), incubated for another 24h, which makes in total 72h of transfection and then subjected to total RNA extraction. Further proceeded with cDNA preparation and then the mRNA levels of TFF1 was analyzed by real-time PCR using Taqman assay and normalized to GAPDH (Fig. 46).

Data analysis showed many interesting candidates, so we subsequently screened again using only the interesting candidates (280 kinases) using the same experimental conditions as the first experiment. We screened again for the third time all the interesting candidates (130 kinases) which had consistently significant changes, again using the same experimental conditions.

Following three rounds of kinase screenings, we were left with 46 potentially interesting candidates (both up- and down-regulated). The identified kinases included MAPK3 and AKT, which phosphorylate ERα at S118 and S167 respectively, confirming that the screen could successfully identify regulators of estrogen-responsive gene expression [253, 259, 271, 329, 330].

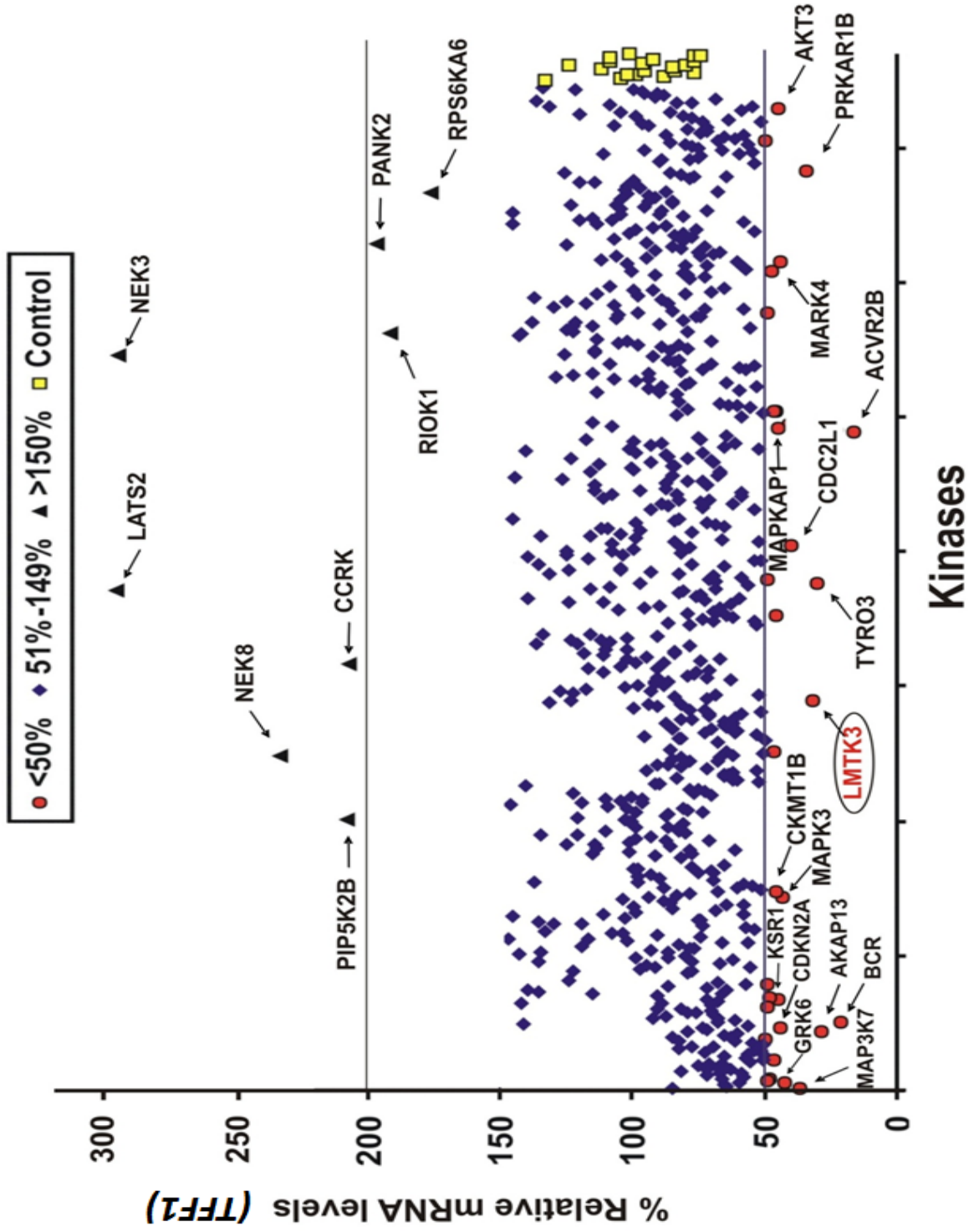

Figure 46: Graphical representation of the entire screening. Graphical representation of the entire screening (691 kinases and the controls). Those highlighted in red circles are Kinases which upon silencing, down-regulated (<50%) TFF1 expression; those highlighted in purple diamond are the kinases which upon silencing, did not result in an altered expression of TFF1; and those highlighted in black triangle are the Kinases which upon silencing up-regulated (>100%) TFF1 expression; finally those in yellow squares are the controls used throughout screening.

3.3.4 Interesting candidates (kinases) upon silencing, up- or down-regulates TFF1 expression

We screened the most interesting candidates (46 kinases) (Fig. 46) with TFF1 again, along with the other estrogen-responsive genes PGR and GREB1. MCF-7 cells were transfected with siRNAs targeting the interesting candidates and appropriate controls were used, which were provided in the library. The estrogen-responsive genes TFF1 (Fig. 47A&B), PGR and GREB1 (data not shown) were used as a read-out. The interesting candidates whose knockdown suppressed TFF1 levels included AKT, MAPK3, ABL1 and BCR which are known regulators of ERα activity and hence were used as positive controls (Fig. 47A). Furthermore, the interesting candidates whose knockdown increased TFF1 levels did not have any positive controls in our screen results as there are not many known kinases which act as a negative regulator for ERα activity. Interestingly, we found a kinase 'LMTK3' whose function was completely unknown and its silencing significantly down-regulates *TFF1* expression which suggests that it could act as positive regulator for ERα activity.

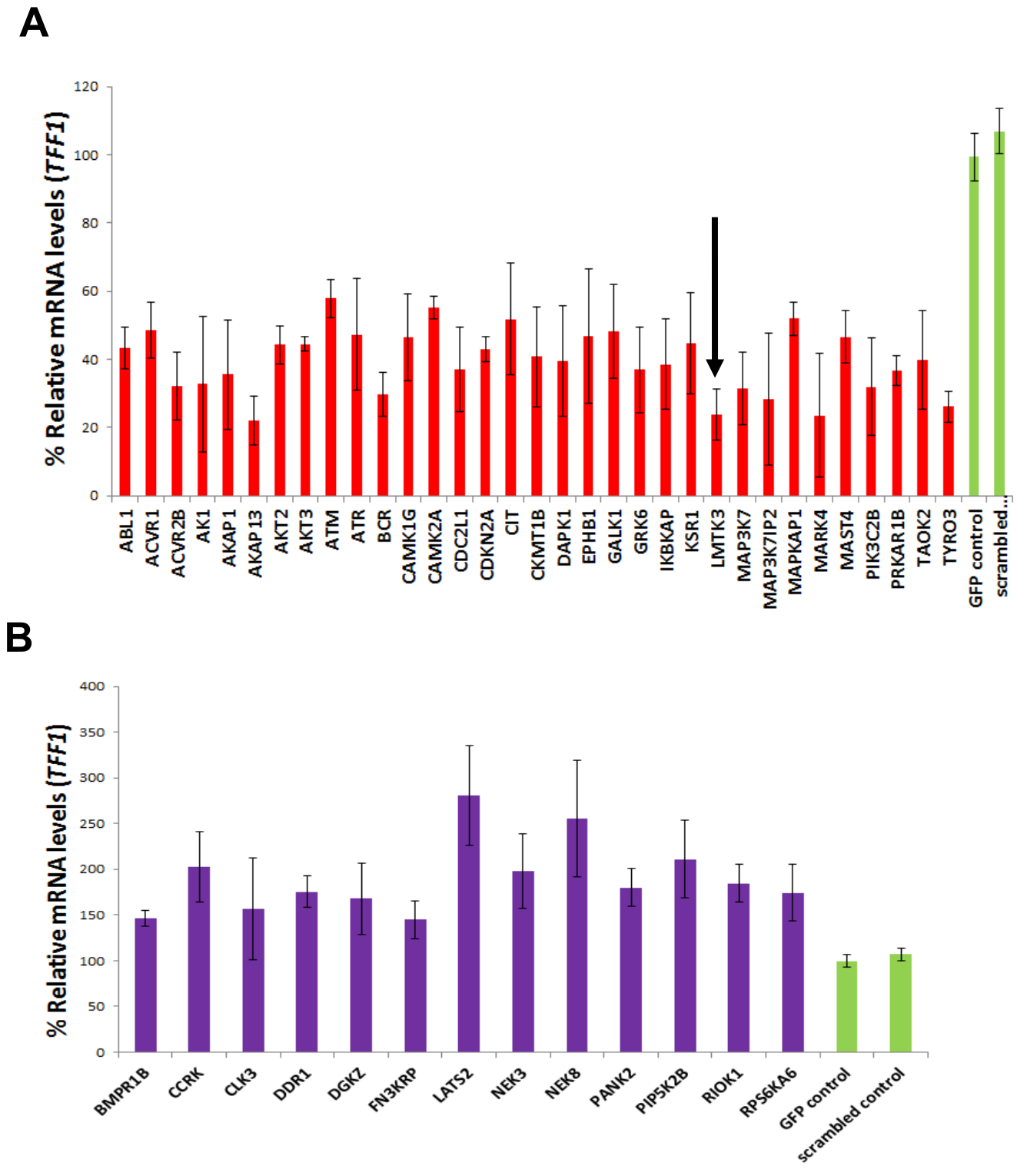

Figure 47: Expression of *TFF1* after silencing of the interesting candidates. MCF-7 cells were plated in 24-well plates and transfected with 20nM of interesting candidate. GFP control and scramble control (negative controls) were used, which were provided in the library. After 48h of transfection, the cells were replaced with the media treated with E2, incubated for another 24h, which makes in total 72h of transfection and then subjected to total RNA extraction. mRNA levels of TFF1 were analysed by real-time PCR using Taqman assay and normalized to GAPDH. **(A)** List of selected kinases upon silencing decreased the TFF1 expression **(B)** List of selected kinases upon silencing decreased the TFF1 expression. Error bars represent standard deviation of three independent experiments is presented.

Table 12: List of selected Kinases regulating ERα chosen from siRNA screening

Group A (up-regulation)		FOLD CHANGE (+)		
siRNA pool	**Gene name**	***TFF1***	***PGR***	***GREB1***
LATS2	large tumor suppressor, homolog 2	2.98	1.34	2.05
NEK3	NIMA (never in mitosis gene a) - related kinase 3	2.96	-	1.14
NEK8	NIMA (never in mitosis gene a) - related kinase 8	2.35	1.19	1.59
PIP5K2B	phosphatidylinositol-4-phosphate 5-kinase, type II, beta	2.11	1.80	1.40
CCRK	cell cycle related kinase	2.03	2.56	1.73
Group B (down-regulation)		FOLD CHANGE (-)		
siRNA pool	**Gene name**	***TFF1***	***PGR***	***GREB1***
ACVR2B	activin A receptor, type IIB	4.65	2.81	1.53
BCR	breakpoint cluster region	3.53	-	-
AKAP13	A-kinase anchor protein 13	3.33	3.22	1.76
TYRO3	TYRO3 protein tyrosine kinase	3.28	5.35	5.59
LMTK3	lemur tyrosine kinase 3	2.96	4.57	2.98
PRKAR1B	protein kinase, cAMP-dependent, regulatory, type I, beta	2.87	-	-
MAP3K7	mitogen-activated protein kinase kinase kinase 7	2.51	1.24	1.71
CDC2L1	cell division cycle 2-like	2.38	1.65	1.45
GRK6	G protein-coupled receptor kinase 6	2.30	1.82	1.67
CDKN2A	cyclin-dependent kinase inhibitor 2A	2.26	1.66	1.17
MARK4	MAP/microtubule affinity-regulating kinase 4	2.25	1.44	1.10
MAPK3	mitogen-activated protein kinase 3	2.25	-	-
CKMT1B	creatine kinase, mitochondrial 1B	2.22	1.09	1.87
KSR1	kinase suppressor of ras 1	2.19	6.94	6.80
AKT3	v-akt murine thymoma viral oncogene homolog 3	2.18	-	-
MAPKAP1	mitogen-activated protein kinase associated protein 1	2.13	-	-

- = not measured

3.3.5 Brief summary of the screening

Overview of the Kinome screen which we performed showing the sequential steps in the selection of hits based on the TFF1 expression. After the third round of screening, in addition to TFF1 expression, we also used the ERα regulated genes PGR and GREB1 as a read-out. We ultimately decided that 'LMTK3' was an exciting kinase on which to focus because of its potential for regulating ERα activity.

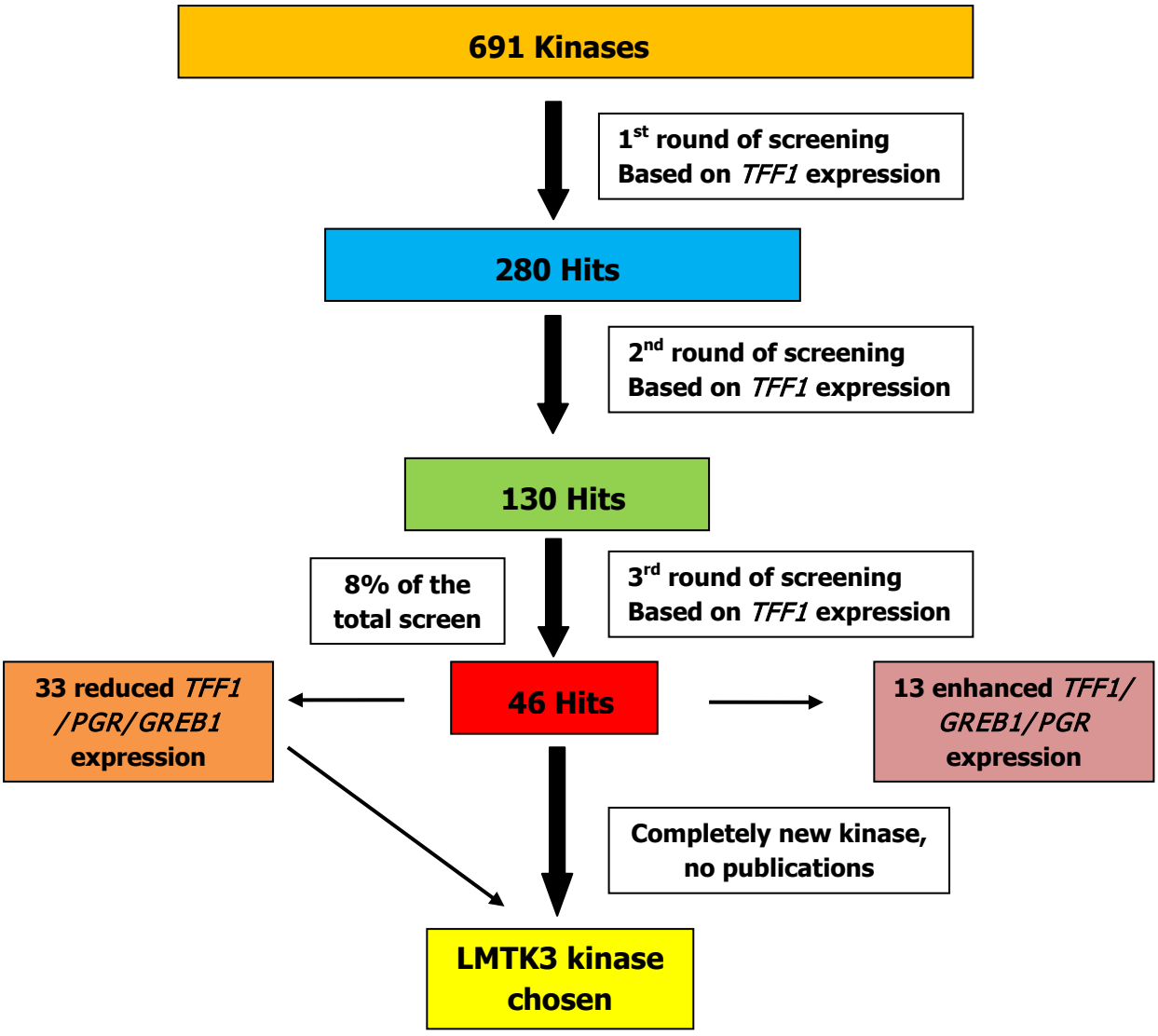

Figure 48: Schematic representation of the screening work.

3.3.6 Validation of LMTK3 as new novel modulator of ERα activity

We wanted to validate that the effect of LMTK3 on ERα activity observed in the screen was not due to an off-target effect of the siRNAs used. Hence we silenced LMTK3 using 4 different siRNAs, all targeting different sites of LMTK3. We transfected MCF-7 cells with 4 different siRNAs targeting LMTK3 and after 48h of transfection the cells were replaced with the media either treated with E2 or untreated and incubated for another 24h. We checked the mRNAs levels of the TFF1, PGR and GREB1 using RT-qPCR. Three siRNAs (siRNA 1, 2 and 3) out of 4 siRNAs supressed the expression of LMTK3 (Fig. 49D) and there was subsequent reduction of estrogen-responsive genes TFF1, PGR and GREB1 (Fig. 49A, B &C). In doing so, we showed that this effect on ERα was not an off-target effect but one associated with LMTK3.

LMTKs are a family of serine-threonine-tyrosine kinases [333-335]. A function has not previously been recognized for LMTK3, although screens have suggested a putative role in the β-catenin pathway [336] and leukemic cell survival [337]. We silenced the other two isoforms of the family LMTK1 and LMTK2, by silencing of either of these two isoforms did not change TFF1 expression levels (Fig. 50A) suggesting that this effect on ERα activity is very specific to LMTK3. We confirmed that silencing actually resulted in supressed expression of LMTK1 and LMTK2 (Fig. 50B).

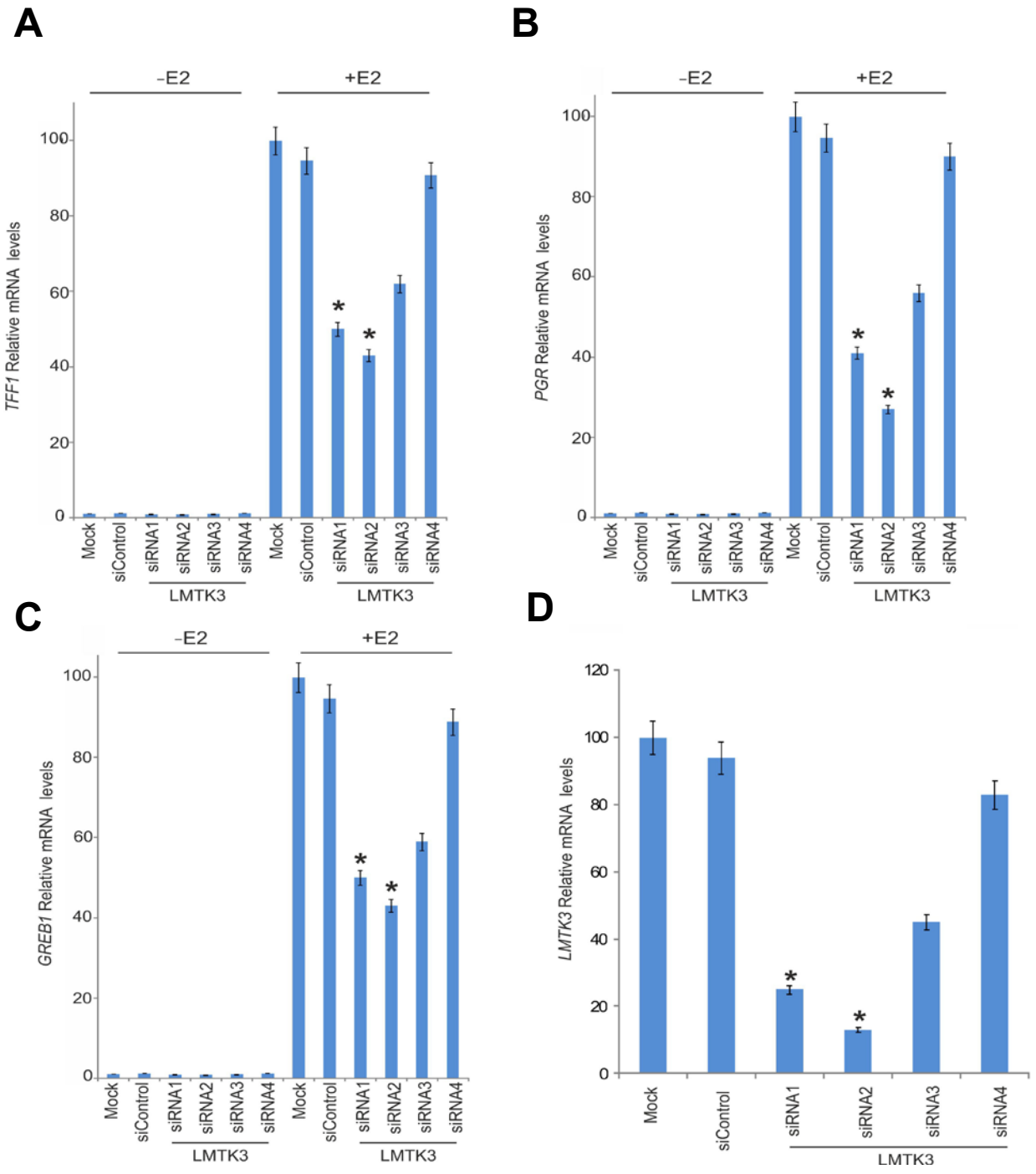

Figure 49: Expression of TFF1, PGR and GREB1 after silencing LMTK3 using four different siRNAs. Validation of the effects of LMTK3 silencing on the E2-induced expression of TFF1, PGR and GREB1 genes. Gene expression of **(A)** TFF1 **(B)** PGR and **(C)** GREB1 in MCF7 cells transfected with 4 individual LMTK3 siRNAs. **(D)** Validation of down-regulation of LMTK3 mRNA levels after treatment with 4 individual LMTK3 siRNAs. Error bars represent standard deviation of three independent experiments is presented. [* $p < 0.05$ compared to siControl (Student's t-test)].

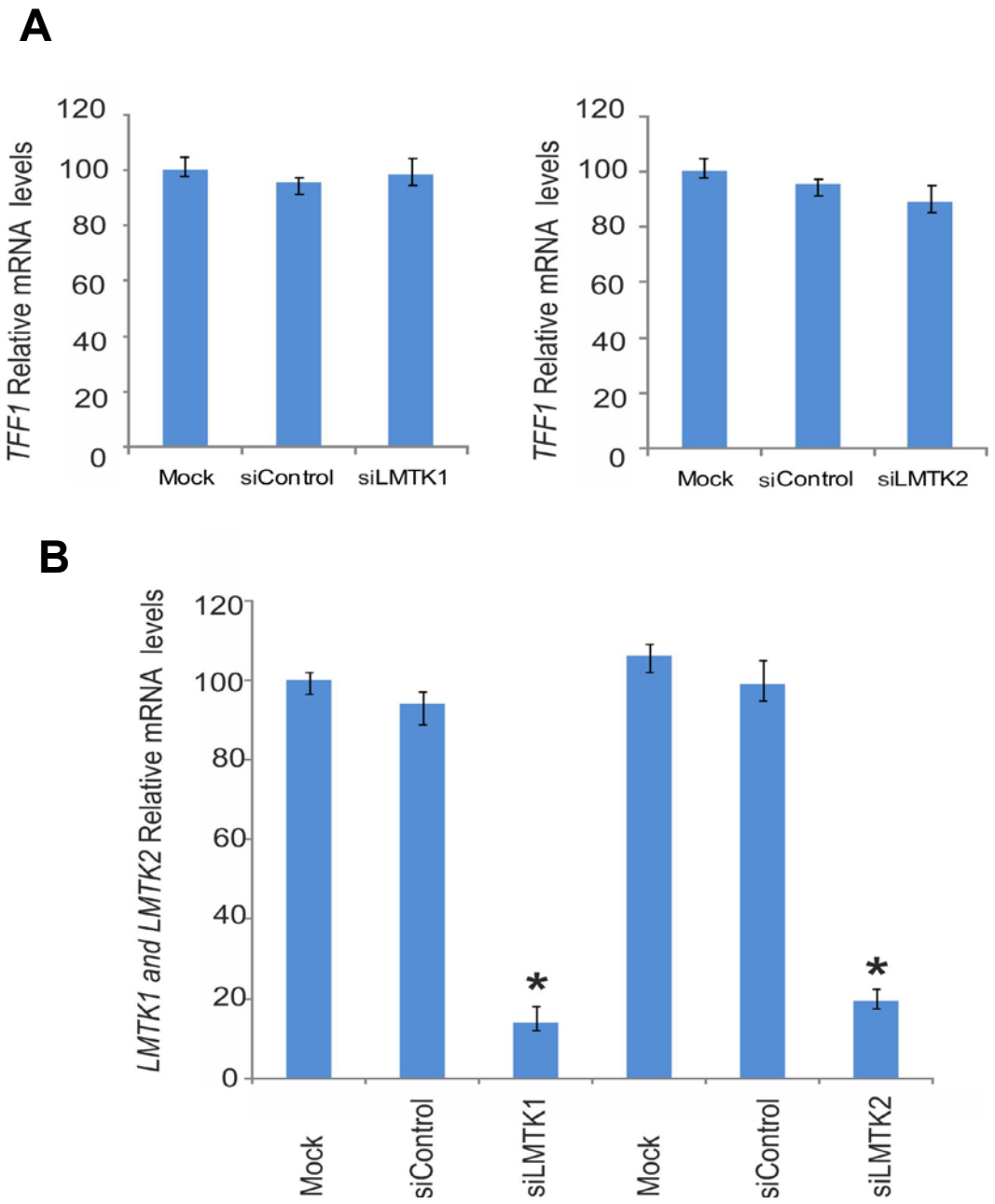

Figure 50: Effects of LMTK1 and LMTK2 silencing on TFF1 expression levels. (A) Gene expression of TFF1 in LMTK1 and LMTK2 siRNA transfected in MCF-7 cells. **(B)** RT-qPCR validation of down-regulation of LMTK1 and LMTK2 mRNA levels after treatment with 20nM of siRNA. GAPDH was used for normalization. Error bars represent standard deviation of three independent experiments is presented. [* $p < 0.05$ compared to siControl (Student's t test)].

We carried out LMTK3 silencing again and also overexpressed the LMTK3 plasmid to validate our findings. We transfected MCF-7 cells with siRNAs targeting LMTK3 and after 48h of transfection the cells were replaced with the media either treated with E2 or untreated and incubated for another 24h. We also transfected MCF-7 cells with 2µg of LMTK3 plasmid and empty vector, after 24h of transfection the cells were replaced with the media either treated with E2 or untreated and incubated for another 24h. We checked the mRNAs levels of the TFF1, PGR and GREB1 using RT-qPCR. LMTK3 silencing consistently inhibited the expression of estrogen-regulated genes effectively (Fig. 51A). Also, overexpression of LMTK3 increased the levels of the estrogen-induced genes confirming that LMTK3 acts as a positive regulator of ERα activity (Fig. 51B).

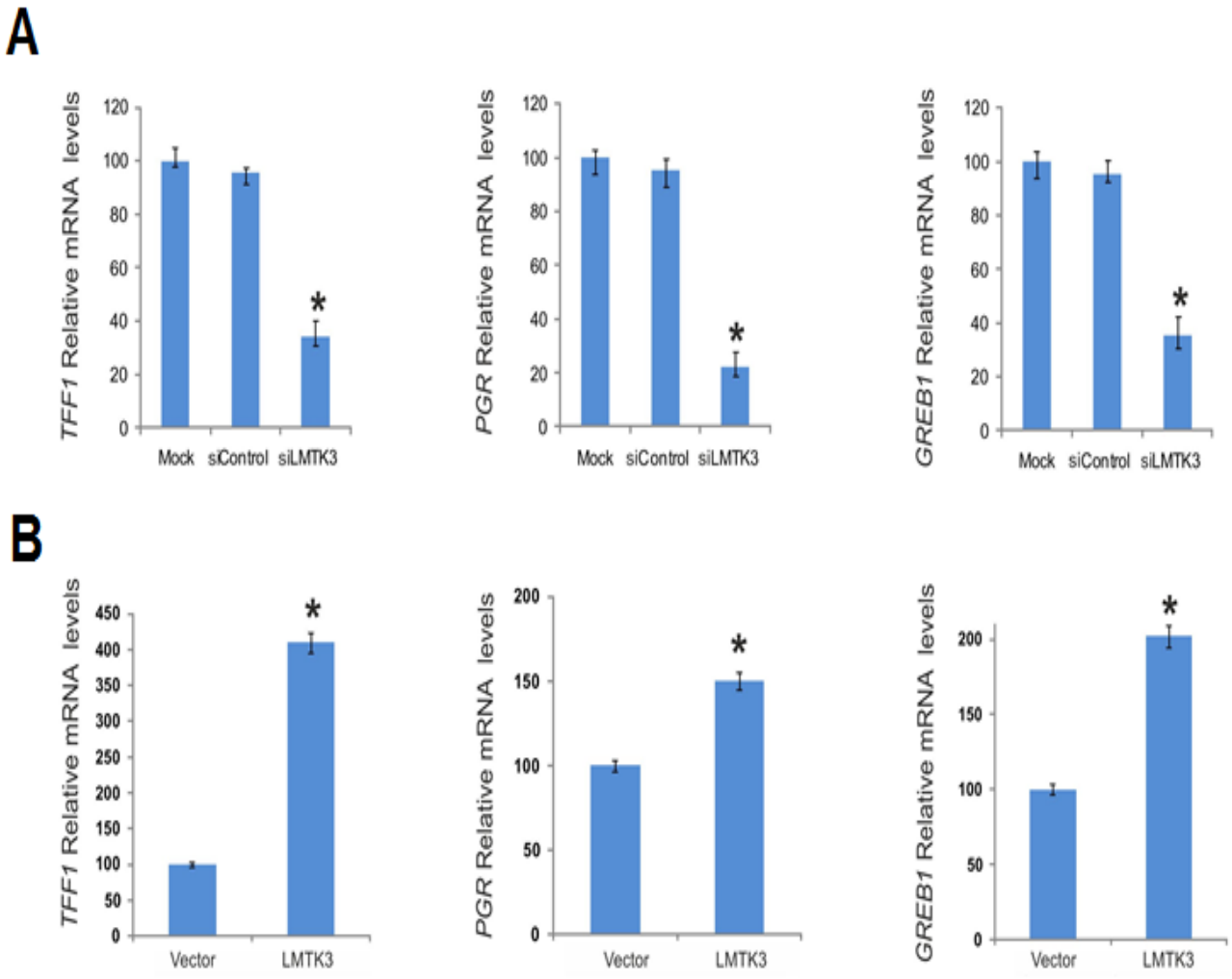

Figure 51: Effects of LMTK3 on expression of estrogen-regulated genes TFF1, PGR and GREB1. (A) Transfection of MCF7 cells with LMTK3 siRNA followed by treatment with E2, significantly inhibited the expression of estrogen-regulated genes (TFF1, PGR and GREB1) while **(B)** over-expression of LMTK3 increased the mRNA levels of these genes. Error bars represent standard deviation of three independent experiments is presented. [* $p < 0.05$ compared to siControl (Student's t test)].

Chapter 4: Discussion

4.0 Discussion

4.1 Regulation of miRNAs by Estrogen receptor

More than 70% of all breast cancers overexpress the estrogen receptor (ER), providing a target for drugs like Tamoxifen, the aromatase inhibitors and other anti-estrogen therapies. Despite most initially responding to such treatment, 40% will subsequently become resistant to this hormone therapy and the patient will relapse [338]. The ERα receptor, a well-studied protein, is known to play an important role in the development of resistance to endocrine therapy [339]. Since their discovery twenty years ago, miRNAs have been proven to be vital modulators of many biological mechanisms. Hence, we decided to focus our research efforts on finding the miRNAs regulated by the estrogen receptor.

We performed a microarray to reveal miRNAs that are regulated by estrogenic response. Prior to the microarray experiment, we optimized the conditions using GREB1 as a positive read-out (Fig.12 A&B). Remarkably, most of the ERα up-regulated miRNAs in the microarray were derived from mir-17-92 and its paralogue clusters mir-106a-363 and mir-106b-25 (Fig. 13 A&B) which has proved to be important in cancer biology [152, 174, 340-347]. The microarray analysis did not reveal any upregulated miRNAs greater than two fold. Validation of some of the microarray results by RT-qPCR showed the precision of the microarray experiment (Fig. 14a-i).

In addition to our research, several other groups have published their findings of estrogenic induction of microRNAs; reporting up and down regulation of several

miRNAs. In summary, the following miRNAs were shown to be up-regulated after early induction (≤ 6h): miR-206 [348], miR-15a, miR-200a, miR-30b and miR -26b [349], miR-424 and miR-760 [350], miR-193b and miR-301b [351]. The miRNAs reported to be down-regulated after early induction (≤ 6h) are the miR-181 family, miR-26 family and miR-23 family [352], miR-424, miR-10b and miR-128 [353], miR-21 [98], miR-221 and miR-222 [354]. These corroborate the findings of our microarray data (Table 10) and suggest the involvement of estrogen receptor in the regulation of miRNA expression.

As our microarray analysis failed to show significant changes in miRNA levels, we were interested to see the expression of their primary transcripts. Using qPCR we showed that mir-17-92, responsible for the production of miR-17, miR-19a, miR-20a, miR-19b-1 and miR-92-1 was upregulated following estrogen treatment, indicating that estrogen plays a role in the regulation of these miRNAs at the transcriptional level (Fig. 15 A&B). To ensure that the changes observed were not due to variations in the expression of the housekeeping gene GAPDH (used for normalization) we used two further housekeeping genes, the small RNAs U6 and U47, that are reported not to change with estrogenic treatment (Fig. 16 A&B). The increase in mir-17-92 expression remained consistent using all three different endogenous controls, hence confirming that this cluster is transcriptionally upregulated by the E2 treatment (Fig 16 A&B).

Having shown that pri-mir-17-92 was considerably more upregulated than the miRNAs it produces, we hypothesized that an inhibition of biogenesis occurred following estrogen mediated transcription (Fig. 17A). In order to define the step(s) of miRNA biogenesis in which this regulation occurs, we then measured

levels of pre-miR-18a and found that DROSHA mir-17-92 cleavage to pre-miR-18a was not the regulatory or 'rate-limiting' step because both are induced at similar levels (Fig. 17B). The primers used to amplify the pre-miR-18a also amplify the pri-miRNAs. To establish that we could distinguish between pri- and pre-miRNA, we used the large RNA fraction to measure mir-17-92 and the small RNA fraction to measure pre-miR-18a. We demonstrated that the levels of the pre-miR-18a were induced similarly to mir-17-92. These data demonstrate that mir-17-92 is induced by the E2-ERα complex, then it is processed by DROSHA releasing the pre-miR-18a, but the passage between pre-miR-18a and miR-18a is attenuated following initial E2 stimulation (Fig. 18).

Such regulation however has been described in let-7 family members during stem cell differentiation. LIN-28 is able to interact with pri-let-7 and/or pre-let-7, impairing its processing [207, 210]. It has also been reported that c-MYC down-regulates let-7 maturation increasing the transcription of LIN-28b in P493-6 B cell lines [355]. Furthermore, it has been shown that when the RNA binding protein KSRP interacts with DICER and promotes the biogenesis of a subset of miRNAs comprising of miR-20a and miR-106a, in both HeLa and NIH-3T3 cells [211]. This suggests that the miRNA biogenic process is finely regulated by specific proteins interacting with either Drosha or Dicer individually, or both together. Since we observed no evidence of estrogen mediated up-regulation of LIN-28 and/or expression of LIN-28b, or evidence of estrogen mediated down-regulation of DICER and/or KSRP in our models, our data indicates that these known RBPs are not responsible (data not shown). Although regulation of pri-mir-17-92-derived microRNAs could not be explained by the candidate factors we tested, the

apparent incidence of regulated miRNA maturation strongly suggests involvement of additional proteins in this process.

Simultaneously, similar research has been undertaken by Bhat-Nakshatri *et al*. [356]. They showed that after stimulation of MCF7 cells for 4 hours with E2, three miRNAs were up-regulated: let-7f, miR-98 and miR-21. They also found that miR-17-5p, a member of the miR-17-92 member was up-regulated, which agrees to our data. Since some of these miRNAs are already known to regulate the estrogen responsive c-MYC, the authors suggested that the estrogenic response is regulated by miRNAs: thereby E2F2 another E2 modulated gene was found to be a new target of the let-7/miR-98 miRNA family [353]. Accordingly, we also found that let-7g, miR-98 and miR-21 were significantly up-regulated by E2 between 3 and 6h after E2 stimulation. In support of their data, we found that miR-27a and miR-27b were among the miRNAs down-regulated by E2 and in addition all the miR-181 family members were included (Table 10).

We found that the modulation of the pri-mir-17-92 by ERα appears to be mediated by the c-MYC oncogene via its direct interaction with the mir-17-92 promoter (Fig. 21 B&C). It has been reported that, in P493-6 B cells, c-MYC directly up-regulates the expression of mir-17-92 and down-regulates the expression of a set of other miRNAs [357]. Since we have not observed any reduction of those miRNAs after estrogenic stimulation, we conclude that the ERα-c-MYC is specifically inducing the expression of mir-17-92 in breast cancer cells.

The importance of miRNA activity in breast cancer biology is also highlighted by the finding that a number of miRNAs show a differential expression between ERα positive and ERα negative breast cancers [358, 359]. We demonstrated that pri-mir-17-92 expression is highly correlated with the level of ERα in breast cancers, and that pre-miR-18a, derived from DROSHA-pri-mir-17-92 cleavage, is also significantly more expressed in ERα positive compared to ERα negative tumours (FiG. 22 A&B). This indicates that a specific increase of this pri-miRNA also occurs in physiologic conditions. It is interesting that miR-18a produced by pri-mir-17-92 is not expressed preferentially in ERα positive tumours (Fig. 22 C&D). This further suggests that ERα positive tumours escape the inhibitory targeting of ERα caused by miRNAs by in turn down-regulating Dicer processing of those miRNAs during tumour progression. Here we demonstrate that the factors implicated in attenuation of miRNA processing are also active in cancer tissues.

Individual miRNAs had smaller effects on targeting the transcripts, but as multiple mature molecules derived from these primary transcripts target ERα and/or AIB1 this increases both the overall level of the miRNAs regulating these two proteins after E2 induction and the effects of silencing; it is known that multiple molecules affecting a single target increase their inhibitory effect [360].

By forming a complex with several co-activators or co-repressors, ERα transcriptionally modulates several genes implicated in cell proliferation and apoptosis such as BCL2, c-MYC and Cyclin D1. SRC1, TIF2 and AIB1 belong to the same family of co-activators that interact and cooperate with ERα in the transcriptional regulation of target genes [361].

miR-17-5p and miR-20a encoded by pri-mir-17-92, and the homologue miR-106b, down-regulate the translation of AIB1 [171, 172, 174]. Given that E2 mediated up-regulation of miR-18a, miR-19b and miR-20b results in down-regulation of ERα (Fig. 25 A&B) and up-regulation of miR-20a, miR-17-5p, miR-106a and miR-20b results in down-regulation of AIB1 (Fig. 27 A&B), we conclude that both primary transcripts are implicated in the regulation of ERα transcriptional activity upon estrogenic stimulation.

Several studies have indicated that after estrogenic induction, both ERα and AIB1 are rapidly down-regulated. This attenuation occurs at transcriptional, post-transcriptional and post-translational levels [75, 76, 78, 79]. We have proved that ERα is regulated by these miRNAs (miR-18a, miR-19b and miR-20b) directly by interacting with 3'UTR of ERα (Fig. 28B). We thereby propose the translational regulation by miRNAs as a further step of ERα transcriptional activity attenuation after estradiol mediated ERα activation. Interestingly, this regulation occurs at a later time and in a negative feedback loop since DICER pri-mir-17-92 processing appeared inhibited after early ERα up-regulation.

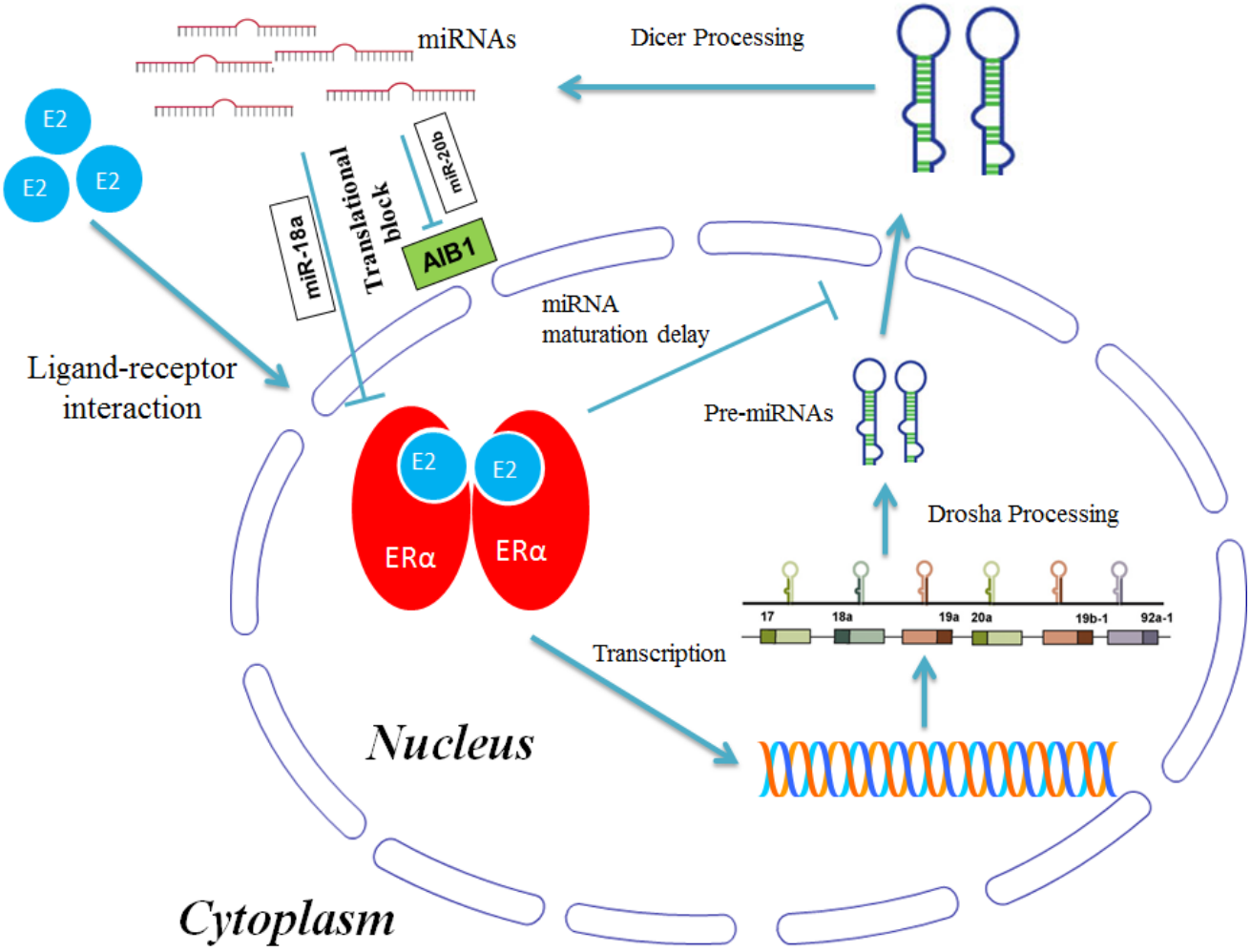

Figure 52: Model of the negative feedback loop and miRNA maturation delay promoted by activated ERα in MCF-7 cells. After ligand binding, ERα induces the transcription of c-MYC that in turn directly activates pri-mir-17-92 transcription. The primary miRNA is promptly cleaved by DROSHA in the nucleus, but the processing from precursor to miRNA is delayed in an E2-dependent manner. The miRNAs derived from the precursors down-regulate ERα and AIB1, therein fine tuning the ERα transcriptional response.

4.2 Possible Estrogen-inducible factor(s) affecting miRNA biogenesis of mir-17-92

We identified the miRNA processing block of the miR-17-92 cluster is due to the involvement of an inhibitor, possibly a molecule that shuttles between the nucleus and cytoplasm (Fig. 31 & 32). By a Mass spectrometry approach (Fig. 33), we tried to identify the RNA binding proteins that could play a role in the processing of microRNAs derived from mir-17-92. It has become more evident that miRNA activity can also be affected by RBPs [362]. Recent studies have introduced a paradigm shift in our understanding of the miRNA biogenesis pathway, which was previously believed to be universal to all miRNAs. It has now been proven that maturation steps of individual miRNAs could be specific for subsets of microRNAs (Table 2). RBPs like hnRNPA1, KSRP, Lin-28 and many more have proved important for the biogenesis of miRNAs and this regulation is crucial, as variations in miRNA expression have been linked to several cancers [196].

Lin-28 was the first negative regulator discovered in miRNA processing but over recent years there have been lot of RBPs involved in negative regulation of the miRNA biogenesis. Interestingly, it has been shown that the RNA methyltransferase, BCDIN3D has a role in miRNA biogenesis by negatively regulating DICER processing of pre-miR-145 [238]. MCPIP1 is an endo-RNase that cleaves the loops of multiple miRNAs, leading to their degradation and hence preventing their processing to mature miRNAs [239, 363].

We screened few RBP candidates, identifying ALY as most interesting candidate (Fig. 36). We showed that ALY did indeed interact effectively with pre-miR-18a *in vitro* (Fig. 40) but further inconsistency in our results suggested that it may not be involved in the miRNA processing. Although the principal function of ALY is to transport mRNAs from the nucleus to the cytoplasm it has been already demonstrated that the nuclear receptor XPO-1 is also implicated in miRNA biogenesis in *C. elegans* and *D. melanogaster* [364]. On the other hand we cannot exclude that the changes we found in the initial experiments could be an indirect effect due to ALY function in the mRNA transport from nucleus to cytoplasm [320, 322]. Therefore silencing ALY could have trapped one of the important proteins involved in miRNA biogenesis in the nucleus, thereby affecting the process. An alternative explanation would be the effect of variation in the snoRNA house-keeping gene U6 as we had different results with different house-keeping genes (u44, u19, u47). In summary, these data suggest that ALY may not be involved in the processing. Hence, we continued our search for other RBPs playing an important role in the processing.

Very recently it was discovered that SND1, a known regulator of edited RNAs, negatively regulates the processing of mir-17-92, particularly the processing of miR-92a in endothelial cells [365]. This was identified by performing mass spectrometry using pre-miR-92a and pulling all of its possible interactors. It would be interesting to see if SND1 has any role in the processing of mir-17-92 via the estrogenic response. On the other hand it has been shown that the estrogenic regulated transcription factor c-Myb positively regulates the

transcription of SND1 in MCF-7 cells in response to E2 treatment [366]. We performed a similar mass spectrometry approach, but using pre-mir-18a instead of pre-mir-92a as bait in an affinity chromatography experiment followed by mass spectrometry. There was no presence of SND1 in our mass spectrometry identified candidates, which could indicate that either our assay was not performed perfectly or that SND1 is not the cause of pri-17-92 miRNA inhibition in MCF-7 cells.

One of the problems we faced was the technical replicates for mass spectrometry experiment. We performed the experiment with appropriate controls and appropriate treatment (estradiol induction) but we lacked technical replicates of the experiment. Although mass spectrometry is specific and accurate, other groups that have identified important microRNA processing factors have used at least three replicates [121, 130, 207, 211] and in some cases they have even used six independent biological replicates [131]. Therefore, we would need to repeat the mass spectrometry experiment with at least three independent replicates and again with all the controls used previously (Fig. 33).

As DICER and TRBP are the most important components for processing precursors to mature miRNAs (Fig. 7), we silenced these two proteins in the MCF-7 and the HEK293 cell lines for at least 3 days as positive control of our procedures. We then checked the levels of three miRNAs (miR-18a, Let-7a, miR-21) derived from 3 different clusters, located on different genomic loci. Interestingly, we did not see low levels of miRNAs after 3 days of silencing (Fig. 52 A&B). The miRNA levels which were detected after Dicer depletion could be

due to either residual Dicer protein after siRNA treatment, or may represent persistence of mature miRNAs over the relatively short time period of these experiments [130]. We further silenced again DICER and TRBP in MCF-7 and HEK293 cells for 9 days (transient transfection with siRNAs every 3 days), we observed much lower levels of miRNAs after 9 days of silencing compared to the silencing after 3 days (Fig. 52 C&D). This data suggests that prolonged silencing of the RBPs is needed to observe the significant changes in miRNA levels and hence longer siRNA silencing techniques are required to see the effect of new factors.

4.3 Kinome Screening

Success of breast cancer treatment has been transformed by therapies targeting the estrogen receptor α (ERα, encoded by ESR1). However, large numbers of women with breast cancer will relapse, emphasizing the need for the discovery of new regulatory targets modulating ERα pathways [2, 29, 367, 368]. ERα phosphorylation is associated with endocrine therapy resistance, particularly the most commonly used drug tamoxifen [328]. Phosphorylation is one of the most important post-translational modifications that the proteins undergo and kinases are the enzymes that catalyse this process; they therefore have a major role in cell function. RNAi screens have become one of the most powerful tools used in drug discovery efforts, particularly in the search for anti-cancer drugs [369].

In response to 17β-estradiol (E2), ERα regulates a wide range of cellular functions by acting as a transcription factor. Apart from the significant widespread role of E2 in various physiological processes, it is also associated with the progression of several types of cancers (breast, prostate, endometrial and ovarian). The proteins encoded by the E2 responsive genes regulate different E2 dependent cellular functions and therefore tight regulation of ERα transcriptional activity is of enormous importance. The regulation of ERα transcriptional activity is highly complex and not yet fully understood. Previously, some kinases have been reported to exert significant impact on ERα transcriptional activity [267, 370, 371]. Previously, RNAi screens have identified important kinases like CDK10 and PDK1 that have a significant role in endocrine resistance [372, 373].

Recently, RNAi screens revealed kinases like EphB4 (as a positive regulator) and JAK2 (as a negative regulator) of ERα transcriptional activity [374, 375].

We decided to do an RNAi screen to identify new kinases that regulate ERα transcriptional activity. We chose the expression of TFF1 as a read-out for the screen as it is the best characterized ERα-regulated gene. TFF1 transcription is strongly controlled by the binding of ERα to an ERE within the TFF1 promoter [376]. Prior to the screening experiment we checked for the silencing efficacy (Fig. 43) and estrogenic induction (Fig. 44).

We performed the siRNA screen to identify kinases that regulate ERα activity, using expression of the estrogen-responsive *TFF1* gene as readout for altered ERα activity (Fig. 46). We identified 5 genes whose knockdown stimulated *TFF1* >100% and 16 genes whose knockdown reduced *TFF1* <50% (Table 12). Two further independent replicate screenings confirmed these findings. Among the other important candidates we chose LMTK3, which upon silencing, down-regulated all the estrogen-responsive genes (TFF1, PGR and GREB1) and it was also a kinase of unknown function.

The off-target effects of siRNA are a common problem (as discussed in chapter 1.10.2), in addition to silencing specific transcripts they can also target other random transcripts [377]. It was shown that the phenotype caused by at least two siRNAs targeting different sites of the transcripts is unlikely to be due to an off-target effect [378]. We wanted to validate the specificity of the effects observed after silencing LMTK3. We used 4 different individual siRNAs that target

LMTK3. We observed 3 out of the 4 siRNAs down-regulated LMTK3 expression and they had significant effects on the estrogen-responsive genes; therefore suggesting that it was not an off-target effect that we observed (Fig. 49). We also overexpressed the LMTK3 protein and it increased the mRNA levels of TFF1, PGR and GREB1 (Fig. 51) revealing LMTK3 as a positive regulator of ERα activity. LMTKs are a family of serine–threonine–tyrosine kinases and have three isoforms LMTK1, LMTK2 and LMTK3; the physiological function for LMTK3 is unknown. Silencing of LMTK1 and LMTK2 did not change the levels of estrogen-responsive gene TFF1 (Fig. 50), suggesting the effect of LMTK3 is very specific within the LMTK family.

Further studies carried out simultaneously by colleagues in our group revealed LMTK3 as a positive regulator of ERα transcriptional activity. It has been shown that LMTK3 knockout led to an 80% reduction of ERα protein. LMTK3 knockout increased the ubiquitination of ERα and, in contrast, the overexpression of LMTK3 phosphorylated ERα, thereby stabilising the ERα protein by protecting it from proteasomal degradation. Silencing of LMTK3 also reduced significantly the protein levels of ERα regulator, FOXO3. Further, Chromatin immunoprecipitation studies revealed LMTK3 increases the binding of FOXO3 to the ESR1 promoter by decreasing the activity of protein kinase C (PKC) and the phosphorylation of AKT [2, 369].

Subsequent studies about LMTK3 expression in breast cancer have revealed both nuclear and cytoplasmic expressions of LMTK3 are implicated with several factors of more aggressive breast cancers. It has been shown that nuclear LMTK3 expression is directly associated with a high tumour grade in both a large

European cohort and an Asian cohort from Singapore. High baseline LMTK3 expression was correlated with decreased overall survival [369, 379]. Recently, Genome-wide studies revealed that LMTK3 has a crucial role in endocrine resistance via different signalling pathways [380]. All of these studies reveal that LMTK3 is a potential therapeutic target and also a new biomarker in ER positive breast cancer.

Chapter 5: Conclusion & Future studies

5.0 Conclusion and Future Studies

In the first part of the project, we identified specific miRNAs (mir-17-92 and paralogue clusters) that are transcribed and then regulated in breast cancer in an estrogen dependent manner. Remarkably, miRNAs belonging to mir-17-92 and its paralogous pri-miRNAs, target and down-regulate ERα, while a subset of miRNAs derived from the paralogue cluster mir-106a-363 inhibits protein translation of AIB1, in an autoregulatory negative feedback loop. Interestingly, we found that levels of pri-mir-17-92 increase earlier than the mature miRNAs derived from it, implicating post-transcriptional regulation of miRNA precursors as a new mechanism of miRNA biogenesis.

Based on these data, we hypothesised that post-transcriptional regulation is a general mechanism of miRNA biogenesis with relevance to breast cancer and an estrogen-inducible mechanism is responsible for the processing inhibition of mature miRNA molecules. Therefore, we aimed to demonstrate the involvement of miRNA binding factors in this process, and also implicate these as potential new therapeutic targets. We performed mass spectrometry to identify the inhibitor that could be involved in the inhibition of Dicer processing. Unfortunately, due to technical and experimental problems we could not find the inhibitory molecule. In the future we would like to repeat the mass spectrometry experiment with at least 3 biological replicates (although ideally more) and further validate the interesting RNA binding proteins (RBPs) revealed from mass spectrometry by screening the potential RBPs based on the interactions discovered from the mass spectrometry experiment. Most importantly, we wish

to elucidate the mechanisms underlying miRNA biogenesis in breast cancer. This is also likely to reveal new RNA binding factors and ultimately these may serve as druggable targets.

We will then investigate the clinical relevance of the mir-17-92 family of miRNAs in breast cancer. We aim to perform an extensive study of the expression of the primary miRNA transcripts, the pre-miRNAs derived from these and their mature molecules in well characterised sets of breast cancer specimens in order to determine their relative expression. We will study whether this ERα regulated miRNAs correlate with outcome including survival, comparing ERα-positive and ERα-negative tumours.

In the second part of the project, we screened the entire kinome and identified a novel kinase, LMTK3 that positively regulates ERα transcriptional activity. Furthermore, research conducted by colleagues from our group showed LMTK3 to be a biomarker in breast cancer as well as being involved in endocrine resistance signalling, and hence a potential future therapeutic target [2, 379, 380]. It will be interesting to explore the mechanism of action of LMTK3 as the functions of LMTK3 were previously unknown. We would like to identify any miRNAs that regulate LMTK3 and vice versa (if any) and examine their effects on the regulatory pathways, using a variety of reporter assays [1]. We plan to explore the post-transcriptional mechanisms of LMTK3 activity on ERα. We will make every effort to determine the exact ERα phosphorylation sites of LMTK3 *in vitro* and *in vivo*, and study their biochemical and functional consequences, including effects on proliferation/apoptosis, ERα stability, function and sub-

cellular localisation. We also aim to identify the LMTK3-ERα interacting domain and investigate the involvement of LMTK3 in the ERα transcriptional machinery (co-activators and co-suppressors). Importantly, we will investigate the clinical relevance of our findings by correlating identified LMTK3-driven ERα phosphorylation sites with single nucleotide polymorphisms (SNPs) in ESR1 and clinical outcomes in large cohorts. This also aims to further validate our existing biomarker data. Together, these aim to establish the mechanism of ERα regulation by LTMK3 in breast cancer, which could be important for understanding endocrine resistance in the clinic.

In addition to LMTK3, our group has also identified LATS2, another kinase detected in our kinase screen, as being an important modulator of ERα activity [381]. Hence, it will be important to research on other kinases identified in the screen (Table 12) and validate their role in the regulation of ERα activity.

This research has identified novel modulators of ERα at both the transcriptional and translational level. We propose that miRNAs derived from mir-17-92 and the novel kinase LMTK3 are potential diagnostic/prognostic biomarkers in ER positive breast cancer. More research is required to validate these observations and their application in clinical practice.

Chapter 6: Appendices

6.0 Appendices

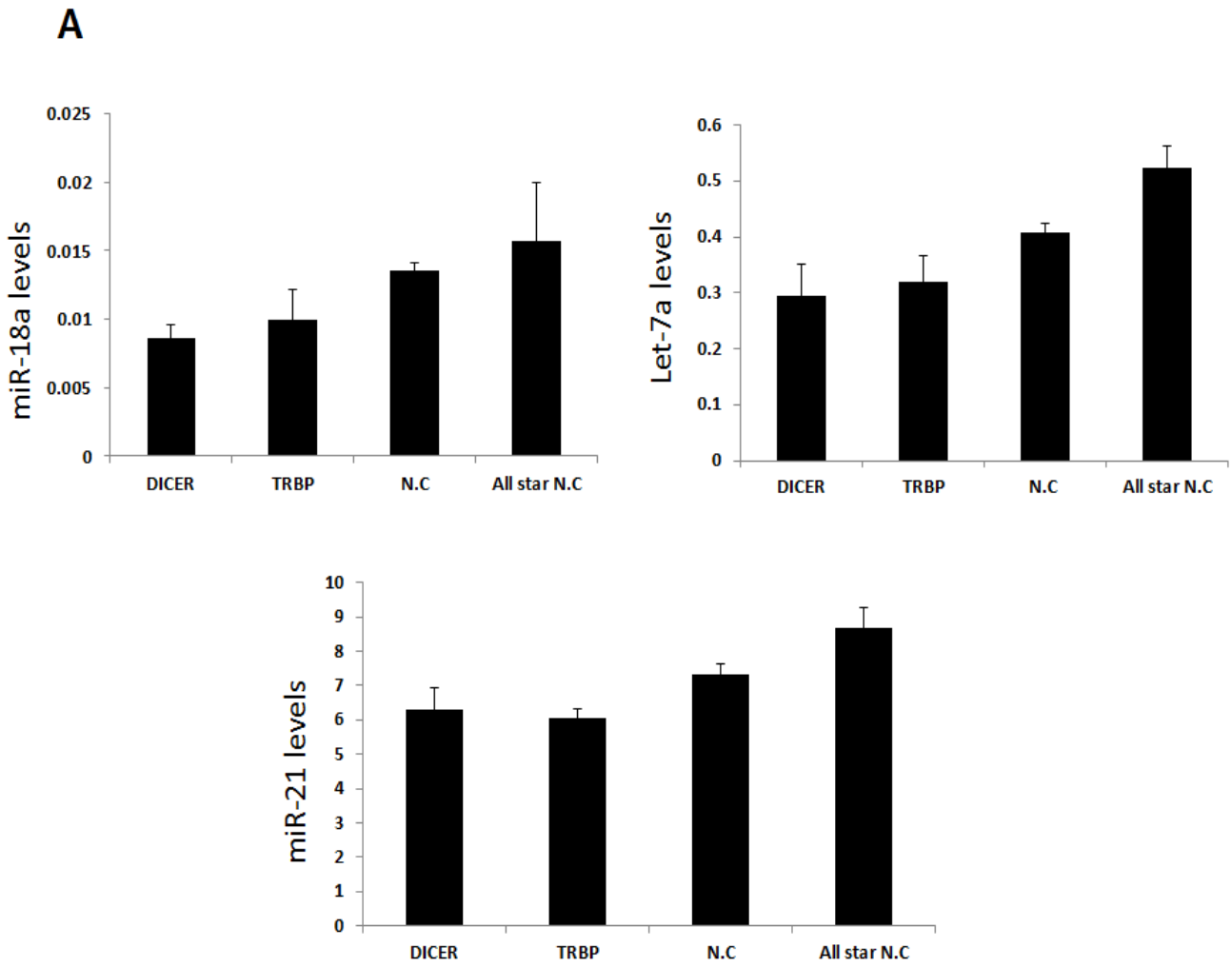

Figure 52: Silencing of DICER and TRBP for 3 days in MCF-7 cells. MCF-7 cells were transfected with 20nM of siRNAs targeting DICER, TRBP and two negative controls (N.C and all star N.C) for 3 days and then subjected to total RNA extraction. **(A)** Expression of miRNA levels of miR-18a, let-7a and miR-21 was analyzed by RT-qPCR using Taqman assay and normalized to U44 snRNA. The mean of two independent experiments are presented, error bars represent s.e.m.

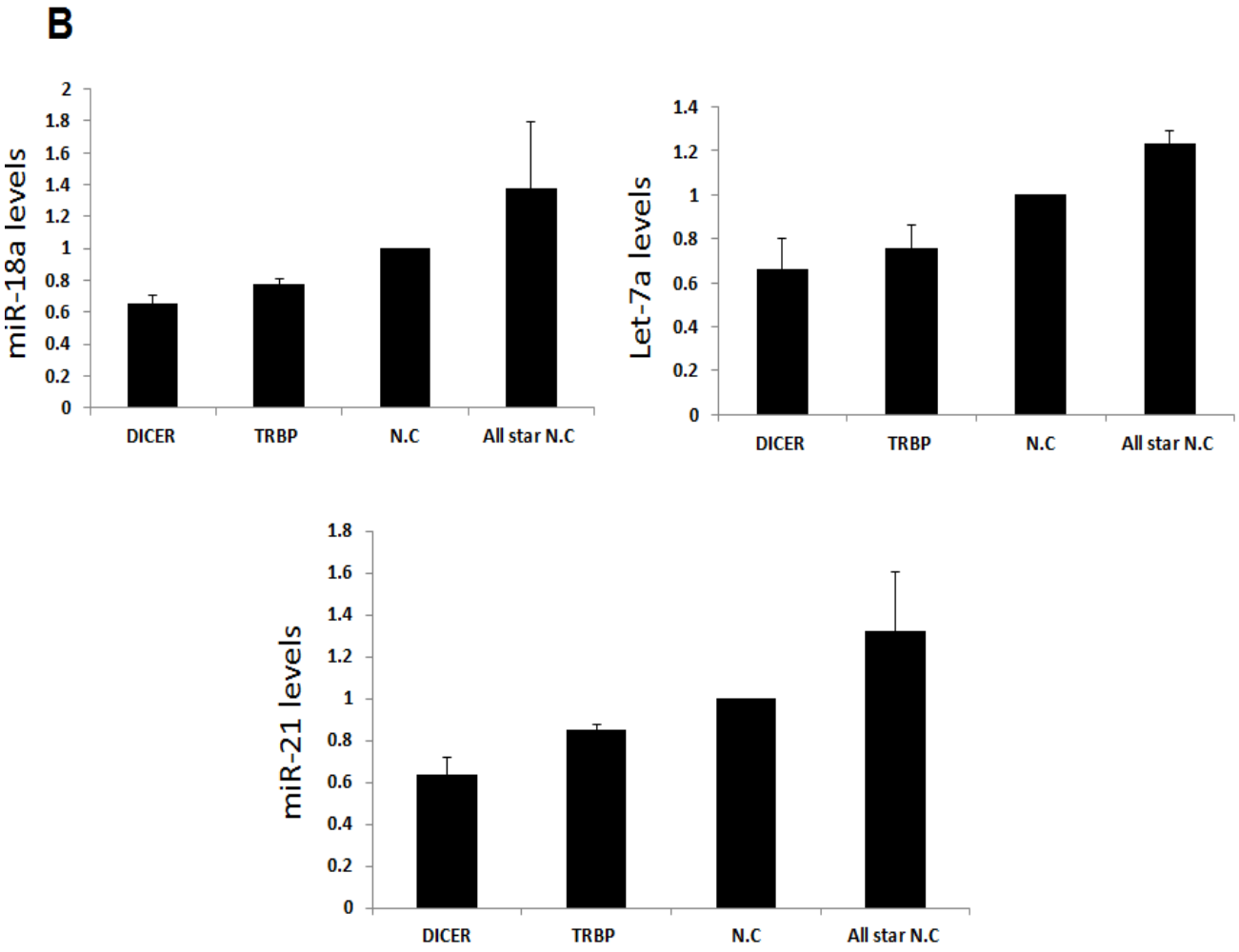

Figure 52: Silencing of DICER and TRBP for 3 days in HEK293 cells. HEK293 cells were transfected with 20nM of siRNAs targeting DICER, TRBP and two negative controls (N.C and all star N.C) for 3 days and then subjected to total RNA extraction. **(B)** Expression of miRNA levels of miR-18a, let-7a and miR-21 was analyzed by RT-qPCR using Taqman assay and normalized to U44 snRNA. The mean of two independent experiments are presented, error bars represent s.e.m.

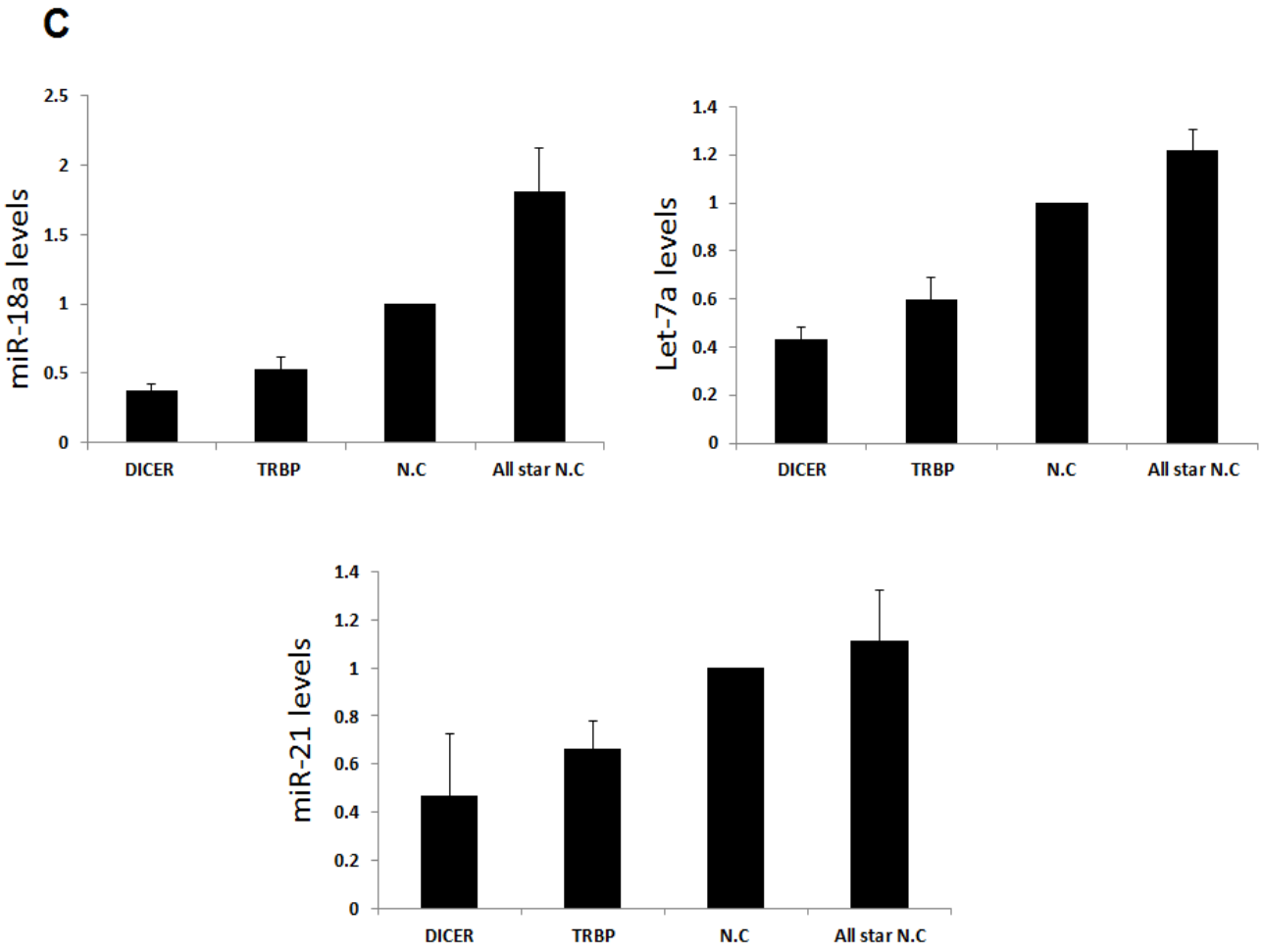

Figure 52: Silencing of DICER and TRBP 9 days in MCF-7 cells. MCF-7 cells were transiently transfected after every 72h with 20nM of siRNAs targeting DICER, TRBP and two negative controls (N.C and all star N.C) for 9 days in total and then subjected to total RNA extraction. **(C)** Expression of miRNA levels of miR-18a, let-7a and miR-21 was analyzed by RT-qPCR using Taqman assay and normalized to U44 snRNA. The mean of two independent experiments are presented, error bars represent s.e.m.

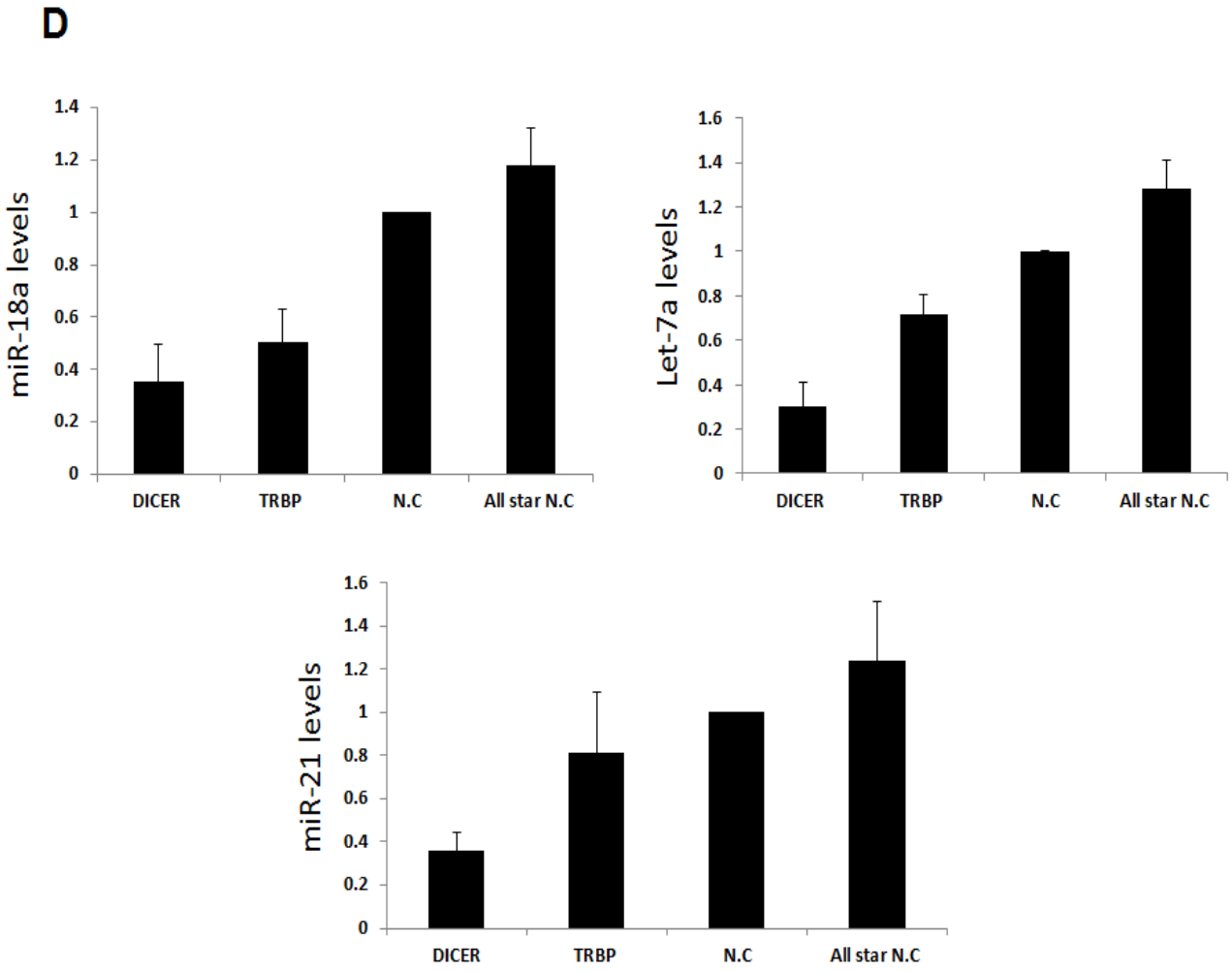

Figure 52: Silencing of DICER and TRBP for 9 days HEK293 cells. HEK293 cells were transiently transfected after every 72h with 20nM of siRNAs targeting DICER, TRBP and two negative controls (N.C and all star N.C) for 9 days in total and then subjected to total RNA extraction. **(D)** Expression of miRNA levels of miR-18a, let-7a and miR-21 was analyzed by RT-qPCR using Taqman assay and normalized to U44 snRNA. The mean of two independent experiments are presented, error bars represent s.e.m.

Chapter 7: References

7.0 References

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The estrogen receptor-α-induced microRNA signature regulates itself and its transcriptional response

Leandro Castellano[a,1], Georgios Giamas[a], Jimmy Jacob[a], R. Charles Coombes[a], Walter Lucchesi[b], Paul Thiruchelvam[a], Geraint Barton[c], Long R. Jiao[d], Robin Wait[e], Jonathan Waxman[a], Gregory J. Hannon[f], and Justin Stebbing[a,1]

[a]Department of Oncology, Cyclotron Building, Hammersmith Hospital Campus, Imperial College, Du Cane Road, London W12 0NN, United Kingdom; [b]Department of Cellular and Molecular Science, Burlington Dane's Building, Hammersmith Hospital Campus, Imperial College, Du Cane Road, London W12 0NN, United Kingdom; [c]Centre for Bioinformatics, Division of Molecular Biosciences, Faculty of Natural Sciences, Biochemistry Building, South Kensington Campus, Imperial College, London SW7 2AZ, United Kingdom; [d]Division of Surgery, Oncology, Reproductive Biology and Anaesthesia, Hammersmith Hospital, Imperial College, Du Cane Road, London W12 0NN, United Kingdom; [e]The Kennedy Institute, Faculty of Medicine, Imperial College, Charing Cross Hospital Campus, London W6 8LH, United Kingdom; and [f]Watson School of Biological Sciences, Howard Hughes Medical Institute, Cold Spring Harbor Laboratory (CSHL), 1 Bungtown Road, Cold Spring Harbor, NY 11724

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Following estrogenic activation, the estrogen receptor-α (ERα) directly regulates the transcription of target genes via DNA binding. MicroRNAs (miRNAs) modulated by ERα have the potential to fine tune these regulatory systems and also provide an alternate mechanism that could impact on estrogen-dependent developmental and pathological systems. Through a microarray approach, we identify the subset of microRNAs (miRNAs) modulated by ERα, which include upregulation of miRNAs derived from the processing of the paralogous primary transcripts (pri-) mir-17–92 and mir-106a-363. Characterization of the mir-17–92 locus confirms that the ERα target protein c-MYC binds its promoter in an estrogen-dependent manner. We observe that levels of pri-mir-17–92 increase earlier than the mature miRNAs derived from it, implicating precursor cleavage modulation after transcription. Pri-mir-17–92 is immediately cleaved by DROSHA to pre-miR-18a, indicating that its regulation occurs during the formation of the mature molecule from the precursor. The clinical implications of this novel regulatory system were confirmed by demonstrating that pre-miR-18a was significantly upregulated in ERα-positive compared to ERα-negative breast cancers. Mechanistically, miRNAs derived from these paralogous pri-miRNAs (miR-18a, miR-19b, and miR-20b) target and downregulate ERα, while a subset of pri-miRNA-derived miRNAs inhibit protein translation of the ERα transcriptional p160 coactivator, AIB1. Therefore, different subsets of miRNAs identified act as part of a negative autoregulatory feedback loop. We propose that ERα, c-MYC, and miRNA transcriptional programs invoke a sophisticated network of interactions able to provide the wide range of coordinated cellular responses to estrogen.

AIB1 | autoregulatory feedback loop | primary transcript | processing

Upon 17-β-estradiol (E2) binding, estrogen receptors (ERs) mediate transcription by interacting directly to specific estrogen response elements (EREs) located in the promoter/enhancer region of its target genes or indirectly by tethering to nuclear proteins, such as AP1 and SP1 transcription factors (2–4). The cellular response to estrogen is highly regulated at multiple levels including transcription, RNA stability, and post-translational modifications (5–8). Following treatment with E2, ERα transcription and mRNA stability is substantially reduced within 1 h of stimulation (7). Furthermore, E2–ERα interactions accelerate receptor degradation through the ubiquitin–proteasome pathway, an effect associated with its major coactivator AIB1 (8).

MicroRNAs (miRNAs) are a class of noncoding short RNAs, 21–24 nucleotides (nt) in length, that play a role in gene regulation. They downregulate expression of their target genes by base pairing to the 3′-UTR of target messenger RNAs (mRNAs) (9). During their biogenesis most miRNAs are transcribed as part of a longer transcript named pri-miRNA (10). These molecules are processed inside the nucleus by DROSHA, producing a pre-miRNA that is a 70-nt "imperfect" stem loop RNA actively transported into the cytoplasm. In the cytoplasm the pre-miRNA is cleaved by DICER, a dual processing event that releases a small double stranded RNA, about 22 nt in length. Here, nuclear processing activity is thought to be regulated at early stages of development and in a variety of tumor cells (11–13). There is also evidence of regulation at the next step, pre-miRNA precursor processing (14, 15). After formation of the small duplex RNA, only 1 strand is loaded onto a miRNA induced silencing complex (RISC). These RISCs, guided by their miRNA, interact with the 3′-UTR or sometimes with the coding region of target mRNAs, inhibiting protein translation or degrading the mRNA target (10).

Substantial data associate changes in miRNA activity with carcinogenesis and progression (16–19). The human mir-17–92 cluster is a polycistronic gene with a chromosomal location 13q31-q32 that encodes 6 miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92–1). Ancient duplications have given rise to 2 mir-17–92 paralogues in mammals: mir-106b-25 and mir-106a-363. Mir-17–92 is thought to be oncogenic in lung cancer and lymphomas (17, 20) or function as a tumor suppressor in breast cancer by downregulating AIB1 and/or cyclin D1 (21, 22). Furthermore, the genomic 13q31 area including mir-17–92 is correlated with loss of heterozygosity in breast cancer (23).

By a genome wide approach, we have elucidated the miRNAs regulated by ERα in breast cancer. Here, we show that among the few miRNAs upregulated by ERα, miR-18a encoded by the pri-mir-17–92, miR-19b encoded by both this primary transcript and its evolutionary paralogue pri-mir-106a-363, and miR-20b encoded by pri-mir-106a-363, downregulate ERα expression at the protein translational level, correlating the induction of these 2 genes during cell proliferation with a negative feedback loop. Remarkably, miR-20b also downregulates and targets the ERα coactivator AIB1. Since ERα can act as a ligand-activated oncogene, we suggest that the pri-mir-17–92 acts as a tumor suppressor in breast cancer, not only by downregulating cyclin D1 and AIB1 via the miR-17/20/106 family, but also by downregulation of ERα by miR-18, miR-19, and miR-17/20/106 members. For the first time we correlate ERα translational control by miRNAs as a further regulatory process involved in ERα transcriptional activity after ligand stimulation.

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[1]To whom correspondence may be addressed: E-mail: l.castellano@imperial.ac.uk or j.stebbing@imperial.ac.uk.

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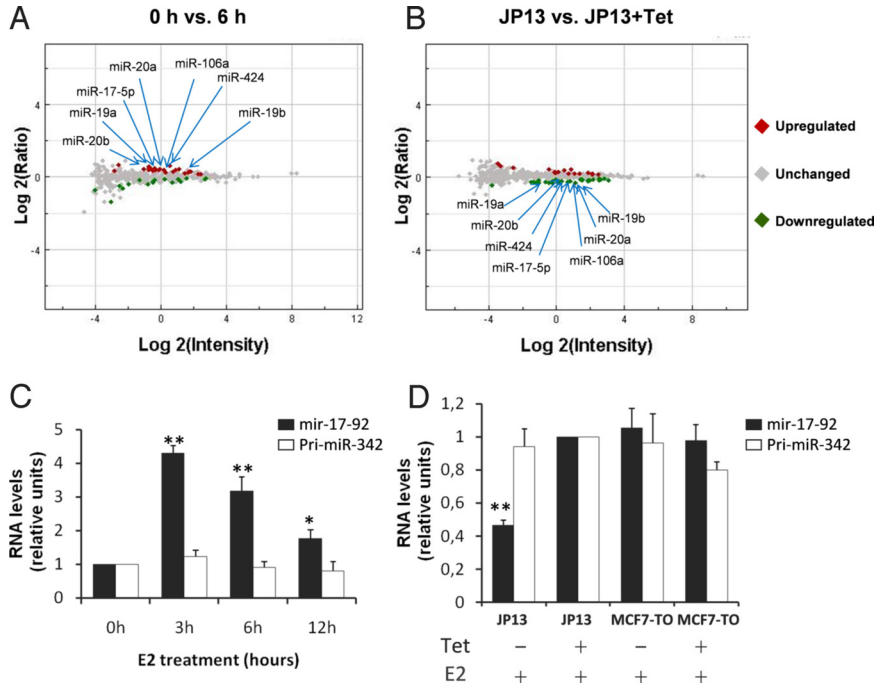

Fig. 1. Pri-mir-17–92 is increased by E2 and decreased by overexpression of PLZF-ERα. (*A*) MCF-7 cell lines underwent E2 stimulation (10 nM) after 72 h of hormone deprivation. After total RNA extraction and labeling we used a microarray platform containing probes for 470 human miRNAs. After hybridization and scanning, raw data were imported into the Rosetta Resolver system for analysis. A $P < 0.01$ was used as cut-off for identification of miRNAs upregulated or downregulated between 0 h versus 6 h. (*B*) JP13 cells were cultured in the presence or absence of Tet for 72 h, followed by the addition of 10 nM E2 for 24 h before microarray analysis. Once again a $P < 0.01$ was used as cut-off for identification of miRNAs downregulated in JP13 + Tet versus JP13 − Tet. (*C*) MCF-7 cells were maintained in DMEM (minus phenol red) supplemented with 10% charcoal-dextran FBS for 3 days and then were either left untreated or treated with 10 nM E2 for the indicated time periods. After total RNA extraction, expression of pri-mir-17–92 and pri-miR-342 was analyzed by RT-qPCR using SYBR green and normalized to *GAPDH*. (*D*) JP13 and MCF-7-TO cells were cultured in the presence or absence of Tet for 72 h, followed by the addition of 10 nM E2 for 24 h. Once again, after total RNA extraction, expression of pri-mir-17–92 and pri-miR-342 were analyzed by RT-qPCR using SYBR green and normalized to *GAPDH*. The mean of 3 experiments each performed in triplicate are presented, error bars represent SEM. For RT-qPCR data, the asterisk indicates $P < 0.05$ in comparison to time 0 h, the double asterisk represents $P < 0.005$ in comparison to time 0 h. *P* values were obtained using a 2-tailed Student's *t*-test.

Results

ERα Induction Reveals pri-mir-17–92 Upregulation. To determine whether ERα regulates the expression of miRNAs, E2 was added to MCF-7 cells and miRNA chip hybridization was performed to elucidate early (0–3 h) and delayed (>6 h) regulation of miRNAs by ERα. As a control we used an MCF-7-TO (MCF-7-Tet-Off)-derived cell line, JP13, that conditionally overexpresses a protein composed of the zinc finger transcriptional repressor PLZF fused to ERα (PLZF-ERα), acting as a dominant-negative that inhibits expression of estrogen-regulated genes and estrogen-stimulated growth of MCF-7 cells (24). Following E2 stimulation and before microarray hybridization, we assessed the reliability of the system using quantitative real time PCR (RT-qPCR) to reveal expression levels of the ERα-regulated gene *GREB1* [supporting information (*SI*) Fig. S1*A* and ref. 25]. *GREB1* expression was reduced in PLZF-ERα cell lines treated without doxytetracycline (Tet) compared to cell lines that do not express the fusion protein (Fig. S1*B*). Although following array analysis we did not reveal any miRNAs with expression changes greater than 2-fold comparing 0 h to 3, 6, and 12 h ($P < 0.05$) (Table S1), we found that those miRNAs that increased following E2 in MCF-7 cells decreased in the JP13-Tet-Off system used as a control (Fig. 1 *A* and *B* and Table S1). The ERα-upregulated miRNAs were generated by the processing of 3 paralogous primary miRNAs: pri-mir-17–92, pri-mir-106a-363, and pri-mir-106b-25 (Fig. 1 *A* and *B*, Table S1 and Fig. S2).

To confirm the change of expression detected by the microarray, we performed RT-qPCR choosing those miRNAs modulated between 1.2- to 2-fold in cells treated with E2 and those repressed similarly by PLZF-ERα in JP13 cell lines. Firstly, we examined the expression of the unprocessed pri-mir-17–92 and family members. Pri-mir-17–92 appeared upregulated within 3 h of E2 treatment reaching a 4- to 5-fold change in comparison to 0 h (Fig. 1*C*) defining it as a new early ERα-regulated gene. Levels of expression were significantly repressed by PLZF-ERα (Fig. 1*D*). Pri-mir-342, a negative control, showed no changes (Fig. 1 *C* and *D*), and we obtained the same results normalizing the value of expression for *GAPDH*, for the snRNA *U6* and for the snoRNA *U47*. We excluded from this analysis the paralogous pri-mir-106b-25 because the fold change of the miRNAs encoded by it were considered too low by our pre-established criteria (Table S1). Furthermore, levels of expression of the pri-mir-106a-363 in MCF-7 cells appeared too low to detect (we were not able to amplify it using 7 different sets of primers from 7 genomic regions). However, it is known that in the P493–6 B cell line that although there is c-MYC-regulated expression of typical miRNAs encoded by both pri-mir-17–92 and pri-mir-106a-363, it is possible to detect the pri-mir-17–92 but not pri-mir-106a-363, indicating that the latter could either be less expressed or alternatively processed more rapidly (26). Furthermore, miR-424, miR-450, and miR-542–3p located within 6 kb of the same genomic region, appeared significantly upregulated by E2 and significantly repressed by PLZF-ERα in a perfectly reciprocal manner (Table S1). We also observed a subset of miRNAs that were subtly downregulated by the E2-ERα complex from 0 to 12 h, and the majority of these belonged to the miR-181 family (Table S1).

Next, we performed RT-qPCR for miR-18a, miR-19a, miR-19b, miR-20a, miR-92 (derived from pri-mir-17–92), miR-19b, miR-20b, miR-92 (from pri-mir-106a-363), miR-424, and miR-181b; the 2 techniques showed an overall correlation (Fig. S3 *A–I* and Table S1). Comparing the low levels of expression of the miRNAs to the higher levels of the pri-miRNA after stimulation, there was negative regulation of miRNA biogenesis, following transcriptional induction by ERα (Fig. 1*C* and Fig. S3).

C-MYC Directly Regulates the pri-mir-17–92 upon Estrogenic Stimulation. It has already been demonstrated that pri-mir-17–92 is transcriptionally regulated by c-MYC in the P493–6 B cell line during the G1–S cellular transition phase (26). Since *c-MYC* mRNA is upregulated by ERα within 1 h of E2 treatment in breast cancer cells (27), c-MYC could contribute to the increased transcription of the pri-mir-17–92 upon E2 stimulation. Interestingly, we observed a half site conserved ERE 70 bp upstream of the c-MYC consensus site (E-box) of the mir-17–92 promoter (Fig. 2*A* and Fig. S4). Using cycloheximide (CHX), we demonstrated that new protein synthesis

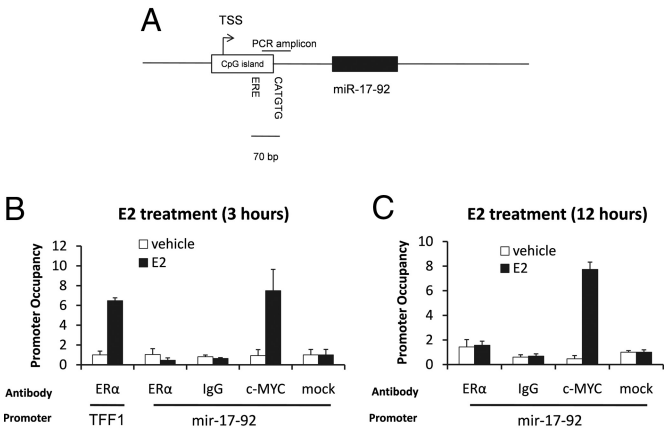

Fig. 2. c-MYC directly regulates the pri-mir-17–92 upon estrogenic stimulation. (*A*) Schematic representation of the mir-17–92 cluster genomic region. Both the c-MYC binding site and a putative ERE half site are indicated. (*B*) MCF-7 cells were maintained in estrogen-free medium for 3 days (starvation) and then either left untreated (vehicle) or treated with 10 nM E2 for 3 h after which ChIP was performed, followed by real time PCR. The c-MYC interaction site genomic region is presented. (*C*) After starvation, MCF-7 cells were treated with E2 for 12 h before ChIP.

is not required exclusively for pri-mir-17–92 expression (Fig. S5) and because it has been demonstrated that estrogen responsive genes can contain both ERα and c-MYC binding elements located within close proximity [13–214 bp within the promoter and regulated by both transcription factors in an E2-dependent manner (28)], we performed chromatin immunoprecipitation (ChIP) assays for both ERα and c-MYC: coprecipitated DNA was analyzed by amplifying the genomic region containing both consensus sites (Fig. 2*A* and Fig. S4) by real time PCR (Fig. 2 *B* and *C*). Although TFF1, a known estrogen-regulated gene, is confirmed here as regulated by ERα (Fig. 2*B*), we observed only c-MYC interacting with the mir-17–92 promoter region analyzed (Fig. 2 *B* and *C*). We demonstrated that c-MYC is recruited to the mir-17–92 promoter in breast cancer cells upon E2 stimulation.

Pri-mir-17–92 Is Negatively Regulated Following DROSHA Cleavage Prolonging miRNA Maturation over Time. Remarkably, the pri-mir-17–92 expression is striking compared to the miRNAs that are produced by its processing (miR-17, miR-18a, miR-19a, miR-19b, and miR-20a), indicative of modulation of miRNAs biogenesis at the posttranscriptional level (Figs. 1*C*, 3*A*, and Fig. S3). A primary transcript undergoes a dual processing event, the first in the nucleus by DROSHA (pre-miRNA production), the second in the cytoplasm by DICER. To define the step(s) of miRNA biogenesis in which regulation occurs, we measured levels of the pri-miR-17–92-derived pre-miR-18a after E2 treatment. DROSHA pri-mir-17–92 cleavage to pre-miR-18a was not a regulatory or "rate-limiting" step here because both were induced at similar levels (Fig. 3*B*). However, the primers used to amplify the pre-miR-18a also amplify pri-mir-17–92. Therefore, to establish that we could distinguish between pri- and pre-miRNA, we stimulated the cell lines with E2 and then separated the small RNA fraction from the large RNA fraction. We used the large RNA fraction to measure pri-mir-17–92 and the small RNA fraction to measure pre-miR-18a (Fig. S6). As a further control we measured the pri-mir-17–92 from the small RNA fraction without obtaining any amplification product. These data demonstrated that pri-mir-17–92 is induced by the E2–ERα complex, then it is processed by DROSHA releasing the pre-miR-18a, but the passage between pre-miR-18a and miR-18a is attenuated until at least 12 h following initial E2 stimulation. Furthermore, using RT-qPCR, we found that both miR-18a and miR-20a mature forms increase their levels of expression from 24 to 72 h after E2 stimulation (Fig. 3 *C* and *D*). Analyzing the levels of the pri-mir-17–92 and the pre-miR-18a from 0 to 72 h, we observed that pri-mir-17–92 is transcriptionally upregulated after 3 h, then DROSHA promptly processes the pri- to the pre-miR-18a, whereas the formation of the mature form from the pre-miR-18a is delayed (Fig. 3*E*). In addition while the miR-18a levels start to increase at 24 h, both pri-mir-17–92 and pre-miR-18a levels decline, indicative of the processing delay we observed (Fig. 3*E*).

Pri-mir-17–92 Expression Is Correlated with ERα Levels in ERα-Positive Primary Breast Cancers. To evaluate ERα modulation of the pri-mir-17–92 at the physiologic level, we examined a correlation between *ERα* mRNA and pri-mir-17–92, and *ERα* mRNA and pre-miR-18a, in breast cancer tissues by RT-qPCR. Levels of pri-mir-17–92 were correlated with *ERα* mRNA in tissues ($r^2 = 0.97$, $P = 0.0002$, Fig. 4*A*), further indicating that ERα regulates the expression of this primary miRNA. However pre-miR-18a was less correlated with ERα ($r^2 = 0.54$, $P = 0.21$, Fig. 4*B*). Next, we addressed whether pre-miR-18a, miR-18a, and miR-20a were differentially expressed in primary breast cancer tissues, comparing the average expression levels between ERα-positive and -negative tumors. Pre-miR-18a levels were significantly higher in ERα-positive tumors (2.52 ± 0.30) compared with negative tumors (0.90 ± 0.08, $P = 0.006$, Fig. 4*C*), supporting our data. Moreover, expression levels of miR-18a showed no significant differences between the 2 groups of samples (Fig. 4*D*), indicating that impaired pre-miR-18a processing to miR-18a occurs in tumors.

MiR-18a, miR-20b, and miR-19b Negatively Modulate the ERα Transcriptional Activity After Estrogen Stimulation. Using the available miRNA target prediction software [TargetScan (29), Pictar (30), and Pita (31)], we observed whether ERα is a potential target of some or all of these miRNAs. Surprisingly, we found that miR-18, miR17/20/106, and miR-19 family members were predicted to target ERα. To experimentally validate this prediction, we chose miR-18a encoded by pri-mir-17–92, miR-19b encoded by both pri-mir-17–92 and the pri-mir-106a-363, and miR-20b encoded by the pri-mir-106a-363 (Fig. S2). First, we addressed whether these miRNAs influence ERα transcriptional activity. MELN cells (MCF-7 cells, stably transfected with a luciferase reporter gene under the control of an ERE using the β-globin promoter) were transfected with pre-miR-18a, pre-miR-20b, and pre-miR-negative control (pre-miR-n.c.). E2-stimulated reporter activity was significantly reduced when MELN cells were transfected with pre-miR-18a and pre-miR-20b, whereas the level of induction was not affected by pre-miR-n.c. (Fig. 5*A*). Remarkably, anti-miR-18a, anti-miR-20b, and anti-miR-19b molecules able to silence their miRNA function significantly increased reporter activity (Fig. 5*B*). The effect of miRNA silencing on luciferase reporter activity was similar to treatment with anti-miR-17–5p, previously reported to reduce the transcriptional activity of ERα by downregulating the coactivator AIB1 (21) (Fig. 5*B*).

Mir-17–5p, miR-106b, and miR-20a are able to negatively regulate AIB1 protein translation by a direct interaction with the 3′-UTR of *AIB1* mRNA (21, 22, 32). Because we observed that miR-17/20/106 and the miR-18 family members potentially target ERα, we evaluated whether the reduction in ERα transcriptional activity induced by miR-20b overexpression was the result of the contemporary negative regulation of AIB1 and ERα and in addition, whether the reduction in ERα transcriptional activity induced by overexpression of miR-18a was the result of a reduction of ERα protein levels. To address if these miRNAs negatively regulate either ERα and/or AIB1, we overexpressed pre-miR-18a, pre-miR-19b, pre-miR-20b, and pre-miR-n.c. and measured protein levels. ERα was markedly reduced by the overexpression of all 3 premiRs analyzed in comparison to either untransfected or pre-miR-n.c.

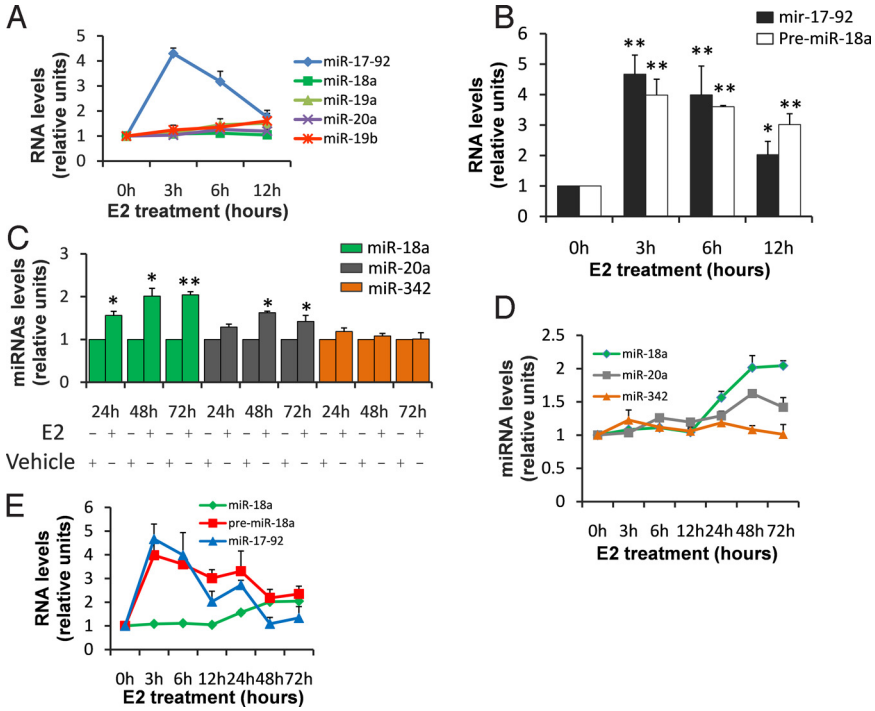

Fig. 3. Pri-mir-17–92 is negatively regulated following DROSHA cleavage prolonging miRNA maturation over time. (*A*) Comparison of the levels of expression between pri-mir-17–92 (normalized to *GAPDH*) and miRNAs encoded from this cluster (normalized to *U47*). The mean of 3 experiments each performed in triplicate are presented, error bars represent SEM. (*B*) After starvation expression levels of both pri-mir-17–92 and pre-miR-18a has been analyzed by RT-qPCR using SYBR green and normalized to *U6* snRNA followed E2 treatment as indicated. The mean of 3 experiments each performed in triplicate are presented, error bars represent SEM. The asterisk indicates $P < 0.05$ in comparison to time 0 h, the double asterisk represents $P < 0.005$ in comparison to time 0 h. *P* values were obtained using a 2-tailed Student's *t*-test. (*C*) After starvation expression levels of miR-18a, miR-20a, and miR-342 were analyzed by RT-qPCR and normalized to *U47* snRNA. The mean of 3 experiments each performed in triplicate are presented, error bars represent SEM. The asterisk indicates $P < 0.05$ in comparison to vehicle treatment, the double asterisk represents $P < 0.005$ in comparison to vehicle treatment. *P* values were obtained using a 2-tailed Student's *t*-test. (*D*) Representation of miR-18a, miR-20a, and miR-342 levels (normalization to *U47* snoRNA) from 0 to 72 h of E2 treatment by RT-qPCR. The mean of 3 experiments each performed in triplicate are presented, error bars represent SEM. (*E*) Representation of the miR-18a (normalization to *U47*), pre-miR-18a and pri-mir-17–92 levels (normalization to *U6*) from 0 to 72 h of E2 treatment. The mean of 3 experiments each performed in triplicate are presented, error bars represent SEM.

transfected cells although the reduction with pre-miR-19b was less pronounced (Fig. 5 *C* and *D*). Furthermore, miR-20b downregulated AIB1 because transfection of pre-miR-20b into MCF7 cells reduced AIB1 protein levels (Fig. 5 *C* and *E*). On the other hand, the transfection of anti-miR-20b increased AIB1 (a dose–response was also observed here; Fig. 5*F*). Because a reduction in either *ERα* or *AIB1* mRNA levels after transfection of precursors was not observed, it appears likely that this regulation occurs at the protein translation step (Fig. S7 *A* and *B*).

To confirm whether these miRNAs directly target ERα, we inserted into the luciferase reporter vector 4 fragments of the 3′-UTR of ERα: the full length (FL, containing all of the putative miRNAs interaction sites), the first half part of the 3′-UTR (ERup for miR-17/20/106 and miR-18), a fragment containing just the putative miR-18 family interaction sites (ER1), and finally a fragment corresponding to the second half section, containing the miR-19 family interaction sites (ER2) (Fig. 6*A*). Transfection of miR-18a, miR-19b, and miR-20b, derived from pri-mir-17–92 and pri-mir-106a-363, were used to investigate direct interactions with the 3′-UTR of ERα constructs and we demonstrated that these miRNAs profoundly downregulate luciferase activity for the constructs containing miRNA interaction sites, but not for the ones in which these sites are absent (Fig. 6*B*). This indicates direct targeting of ERα by a number of miRNAs derived from these paralogous primary miRNAs. We did not observe any downregulation of luciferase reporter activity upon miR-17–5p overexpression, according to a recent report (33).

Discussion

In this study we were able to classify miRNAs upregulated by estrogen as the members encoded by the paralogous transcripts pri-mir-17–92 and pri-mir-106a-363. For individual miRNAs small changes were observed, but as multiple mature molecules derived from these primary transcripts target ERα and/or AIB1 this increases both the overall level of the miRNAs regulating these 2 proteins after E2 induction and the effects of silencing; it is known that multiple molecules affecting a single target increase their inhibitory effect (34).

Changes in pri-mir-17–92 were significantly greater than the miRNAs derived from it, implicating inhibition during miRNA biogenesis: DROSHA cleavage of pri- to pre-miRNAs occurred rapidly, indicating that this step is not rate limiting (Fig. S8). Such regulation however has been described regarding let-7 family members during stem cell differentiation: LIN-28 is able to interact with pri-let-7 and/or pre-let-7 impairing its processing (12, 15). It has also been reported that c-MYC downregulates let-7 maturation increasing the transcription of LIN-28b in P493–6 B cell lines (35). Furthermore, it has been shown that the RNA binding protein KSRP, interacting with DICER, promotes the biogenesis of a subset of miRNAs comprising miR-20a and miR-106a in both HeLa and NIH 3T3 cells (36). Because we did not observe any estrogen-mediated upregulation of LIN-28 and/or any expression of LIN-28b, or any estrogen-mediated downregulation of DICER and/or KSRP in our models, this indicates that these factors are not responsible. Our data indicate that pri-mir-17–92 (not only let-7), is regulated after induction. Additionally, many expression studies note discordance in the levels of mature miRNAs derived from polycistronic precursors. Although regulation of pri-mir-17–92-derived microRNAs could not be explained by the candidate factors we tested, the apparent prevalence of regulated miRNA maturation strongly suggests involvement of additional RNA binding proteins in this process.

The importance of miRNA activity in breast cancer biology is also highlighted by the finding that a number of miRNAs show a differential expression between ERα positive and ERα negative breast cancers (37, 38). We demonstrated that pri-mir-17–92 expression is highly correlated with the level of ERα in breast cancers, and that pre-miR-18a derived from DROSHA-pri-mir-17–92 cleavage is also significantly more expressed in ERα-positive compared to ERα-negative tumors. This indicates that a specific increase of this pri-miRNA also occurs in physiologic conditions. It is interesting that miR-18a produced by pri-mir-17–92 is not expressed

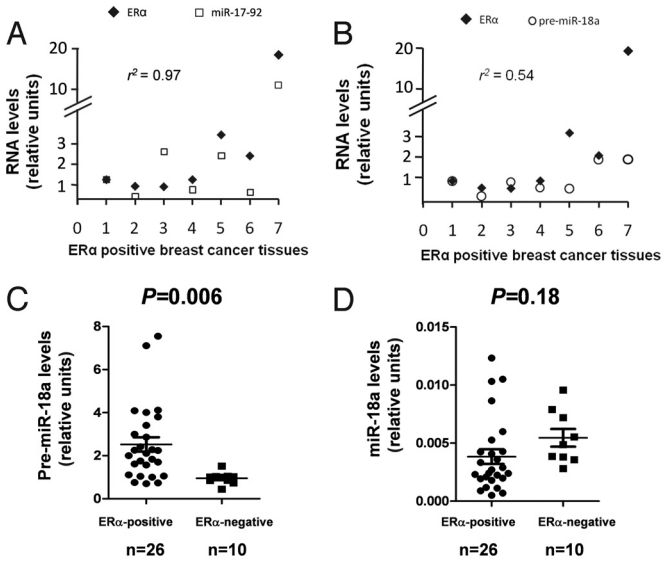

Fig. 4. ERα modulates pri-mir-17–92 in breast cancer tissues. (*A*) Expression levels of *ERα* and pri-mir-17–92 (Pearson correlation 0.97) or (*B*) pre-miR-18a (Pearson correlation 0.54) was measured by RT-qPCR in ERα-positive breast cancers. (*C*) RT-qPCR showed that expression levels of pre-miR-18a are significantly higher in ERα-positive than in ERα-negative tumors (unpaired, 2-tailed Student's *t*-test $P = 0.006$). Error bars represent SEM. (*D*) RT-qPCR showed that expression levels of miR-18a are not different between ERα-positive and ERα-negative tumors (unpaired, 2-tailed Student's *t*-test $P = 0.18$). Error bars represent SEM.

preferentially in ERα-positive tumors. This further suggests that ERα-positive tumors escape the inhibitory targeting of ERα caused by miRNAs by in turn downregulating DICER processing of those miRNAs during tumor progression. Here we demonstrate that the factors implicated in attenuation of miRNA processing are also active in cancer tissues themselves.

The modulation of the pri-mir-17–92 by ERα appears mediated by the c-MYC oncogene by its direct interaction with the mir-17–92 promoter. It has been reported that c-MYC directly downregulates the expression of a set of miRNAs in B cells (39). Because we have not observed any reduction of those after estrogenic stimulation, we conclude that the upregulation of pri-mir-17–92 through ERα-c-MYC is specific to breast cells.

By forming a complex with several coactivators or corepressors, ERα transcriptionally modulates several genes implicated in cell proliferation and apoptosis such as BCL2, c-MYC, and cyclin D1. AIB1, SRC1, and TIF2 belong to the same family of coactivators that interact and collaborate with ERα in the transcriptional regulation of target genes (40). MiR-17–5p and miR-20a encoded by pri-mir-17–92, and the homologue miR-106b, downregulate the translation of AIB1 (21, 22, 32). Because following E2-mediated upregulation: (*i*) miR-18a, miR-19b, and miR-20b downregulate ERα and (*ii*) miR-20a, miR-17–5p, miR-106a, and miR-20b downregulate AIB1, we conclude that both primary transcripts are implicated in the regulation of ERα transcriptional activity upon estrogenic stimulation. Several studies have indicated that after estrogenic induction, both ERα and AIB1 are rapidly downregulated. This attenuation occurs at transcriptional, posttranscriptional, and posttranslational levels (5–8). We propose here the translational regulation by miRNAs as a further step of ERα transcriptional activity attenuation after estradiol-mediated ERα activation. Interestingly, this regulation occurs especially at a later time and in a negative feedback loop because DICER pri-mir-17–92 processing appeared inhibited after early ERα upregulation (Fig. S8).

Methods

MiRNA Microarray. Isolated RNA was labeled using the Agilent labeling kit following the manufacturer's instruction (Agilent Technologies). The Agilent human (V1) miRNA microarray platform, containing probes for 470 human (and 64 viral miRNAs from the Sanger database v9.1), was used to perform miRNA expression profiling.

RT-qPCR Assay. For RT-qPCR assays, cDNA was synthesized from 1 μg of purified Dnase-treated RNA by the SuperScript III First-Strand cDNA synthesis system (Invitrogen); RT-qPCR was performed on a 7900HT Thermocycler using the Power SYBR green PCR master mix (both from Applied Biosystems). For detection of mature miRNAs, the TaqMan MicroRNA assay kit (Applied Biosystems) was used. Sequences of primers used are provided in Table S2.

ChIP. Cross-linked chromatin was prepared from MCF-7 cells as described previously with minor modifications (43). Aliquots of 20 μg were incubated

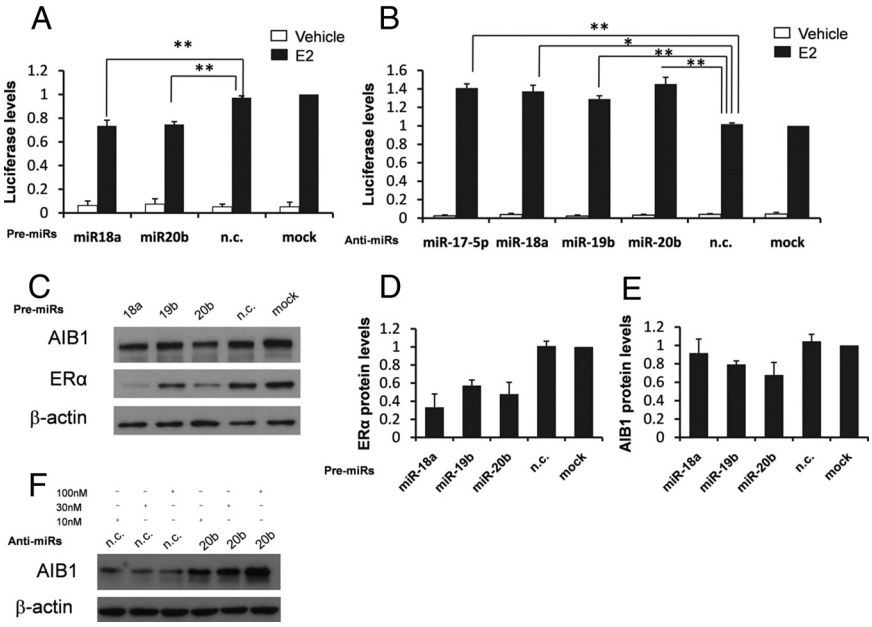

Fig. 5. MiR-18a, miR-19b, and miR-20b suppress ERα-mediated signaling. (*A*) Luciferase activity in MELN cells untransfected or transiently transfected for 48 h with pre-miR-18a, pre-miR-20b, and pre-miR-n.c. in the absence or presence of 10 nM of E2 for 24 h. (*B*) Luciferase activity in MELN cells transiently transfected for 48 h with anti-miR-17–5p, anti-miR-18a, anti-miR-19b, anti-miR-20b, anti-miR-n.c. or untransfected in the absence or presence of 10 nM of E2 for 24 h. (*C*) Western blot showing ERα, AIB1, and β-actin in MCF-7 cells untransfected or transiently transfected with pre-miR-18a, pre-miR-19b, pre-miR-20b, and pre-miR-n.c. (*D*) Densitometric analysis of ERα Western blot shown in *C* normalized to β-actin. (*E*) Densitometric analysis of AIB1 Western blot (shown in *C* normalized to β-actin. The mean of 3 independent experiments are presented, error bars represent SEM. (*F*) Western blots showing AIB1 and β-actin in MCF-7 cells transfected with anti-miR-n.c. and anti-miR-20b at 10, 30, and 100 nM concentrations. One representative experiment from 3 independent experiments is shown.

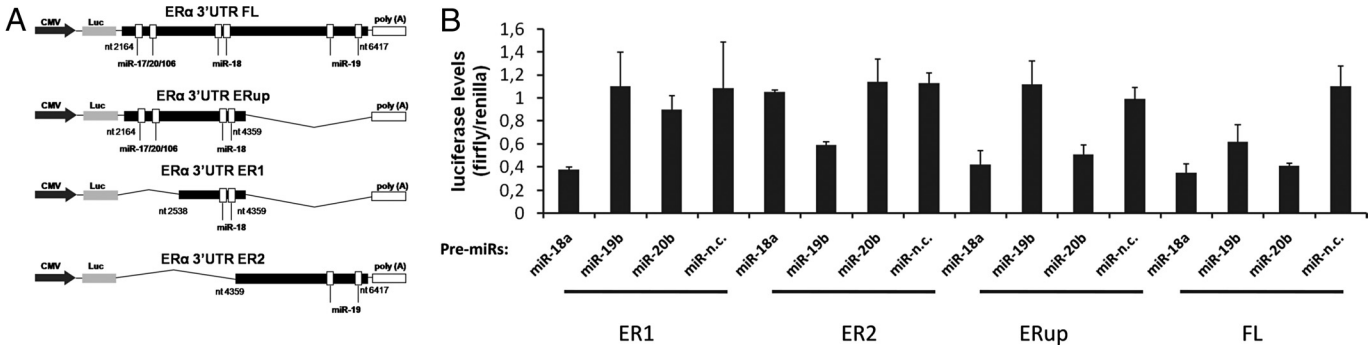

Fig. 6. ERα is directly regulated by miRNA-3′-UTR interaction. (*A*) Representation of the 4 different lengths of ERα 3′-UTR cloned in the pMIR-REPORT lucifearse vector and miRNAs interaction sites. (*B*) Luciferase activity from cells cotransfected with pre-miR-18a, pre-miR-19b, pre-miR-20b, pre-miR-n.c., and different lengths of DNA fragments corresponding to the ERα 3′-UTR. *Firefly* luciferase was normalized for transfection levels to *Renilla* luciferase as indicated in experimental procedures.

overnight with 2 μg of c-Myc (sc-764) and ERα (sc-543) antibodies (Santa Cruz Biotechnology) or without (mock controls) in a total volume of 1 mL and immunoprecipitated. Triplicate samples of 5 μL of immunoprecipitated genomic DNA were amplify by real time PCR. Values are expressed as fold of enrichment with respect to input DNA. Primer sequences used in this assay are listed in Table S2.

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LETTERS

Kinome screening for regulators of the estrogen receptor identifies LMTK3 as a new therapeutic target in breast cancer

Georgios Giamas[1], Aleksandra Filipović[1], Jimmy Jacob[1], Walter Messier[2], Hua Zhang[1], Dongyun Yang[3], Wu Zhang[3], Belul Assefa Shifa[1], Andrew Photiou[1], Cathy Tralau-Stewart[4], Leandro Castellano[1], Andrew R Green[5], R Charles Coombes[1], Ian O Ellis[5], Simak Ali[1], Heinz-Josef Lenz[3] & Justin Stebbing[1]

Therapies targeting estrogen receptor α (ERα, encoded by *ESR1*) have transformed the treatment of breast cancer. However, large numbers of women relapse, highlighting the need for the discovery of new regulatory targets modulating ERα pathways[1–5]. An siRNA screen identified kinases whose silencing alters the estrogen response including those previously implicated in regulating ERα activity (such as mitogen-activated protein kinase and AKT). Among the most potent regulators was lemur tyrosine kinase-3 (LMTK3), for which a role has not previously been assigned. In contrast to other modulators of ERα activity, LMTK3 seems to have been subject to Darwinian positive selection, a noteworthy result given the unique susceptibility of humans to ERα$^+$ breast cancer. LMTK3 acts by decreasing the activity of protein kinase C (PKC) and the phosphorylation of AKT (Ser473), thereby increasing binding of forkhead box O3 (FOXO3) to the *ESR1* promoter. LMTK3 phosphorylated ERα, protecting it from proteasomal degradation *in vitro*. Silencing of LMTK3 reduced tumor volume in an orthotopic mouse model and abrogated proliferation of ERα$^+$ but not ERα$^-$ cells, indicative of its role in ERα activity. In human cancers, LMTK3 abundance and intronic polymorphisms were significantly associated with disease-free and overall survival and predicted response to endocrine therapies. These findings yield insights into the natural history of breast cancer in humans and reveal LMTK3 as a new therapeutic target.

More than two-thirds of breast tumors express ERα (ref. 2), and patients with ERα$^+$ disease respond to antiestrogens (tamoxifen), estrogen withdrawal (aromatase inhibitors) and direct targeting of the receptor (fulvestrant)[1]. The introduction of these treatments has had a profound impact on patient survival[6]. However, resistance to these therapies is common, and *in vitro* evidence points to the role of ERα phosphorylation[3] in the development of endocrine resistance[4,5]. To identify kinases that regulate ERα activity, we performed a whole human kinome siRNA screen using expression of the estrogen-responsive *TFF1* gene, encoding trefoil factor-1, as a read-out for altered ERα activity in the presence of estradiol (E2 (ref. 7)) (**Supplementary Fig. 1**). We identified five genes whose knockdown resulted in a >100% increase in *TFF1* expression and 16 genes whose knockdown reduced *TFF1* expression by <50% (**Fig. 1a**). Two further independent replicate screenings confirmed these findings (data not shown). The identification of the kinases mitogen-activated protein kinase-3 (MAPK3) and AKT, which phosphorylate ERα at Ser118 and Ser167, respectively[8–12], confirmed the screen could successfully identify regulators of estrogen-responsive gene expression.

We subsequently measured the expression of two other ERα regulated genes (*PGR*, encoding progesterone receptor, and *GREB1*, encoding growth regulation by estrogen in breast cancer-1)[13] and two control genes (*GAPDH*, encoding glyceraldehyde 3-phosphate dehydrogenase, and *MCL1*, encoding myeloid cell leukemia sequence-1). Two of the five genes (*LATS2* (encoding large tumor suppressor, homolog 2) and *CCRK* (encoding cell cycle–related kinase)) whose downregulation upregulated *TFF1* also upregulated *PGR* or *GREB1* >100% (group A, upregulated), and three of the 13 kinases (*TYRO3* (encoding protein tyrosine protein kinase receptor 3), *LMTK3* and *KSR1* (encoding kinase suppressor of ras 1)) that downregulated *TFF1* expression were also able to downregulate the expression of both *PGR* and *GREB1* >50% (group B, downregulated) (**Supplementary Table 1**), whereas the expression of *GAPDH* and *MCL1* did not change, indicating that the effects were E2 treatment dependent.

To prioritize among the kinases whose silencing downregulated the activity of ERα, we asked whether any of the candidate proteins showed evidence of positive selection as measured by analyzing changes in synonymous versus nonsynonymous genomic alterations. It is well established that humans and the great apes (especially chimpanzees) differ in their susceptibilities to epithelial neoplasms, including breast cancer[14–18] possibly resulting from recent evolutionary events reflected in the adaptive profile of genes that have a regulatory role in estrogenic signaling. Of those genes that we found to

[1]Department of Surgery and Cancer, Imperial College London, Hammersmith Hospital Campus, London, UK. [2]Evolutionary Genomics, Inc., Lafayette, Colorado, USA. [3]Division of Medical Oncology, University of Southern California, Norris Comprehensive Cancer Center, Keck School of Medicine, Los Angeles, California, USA. [4]Drug Discovery Centre, Imperial College London, Biochemistry Building, South Kensington Campus, London, UK. [5]Histopathology, School of Molecular Medical Sciences, University of Nottingham and Nottingham University NHS Hospital Trust, Nottingham, UK. Correspondence should be addressed to G.G. (g.giamas@imperial.ac.uk) or J.S. (j.stebbing@imperial.ac.uk).

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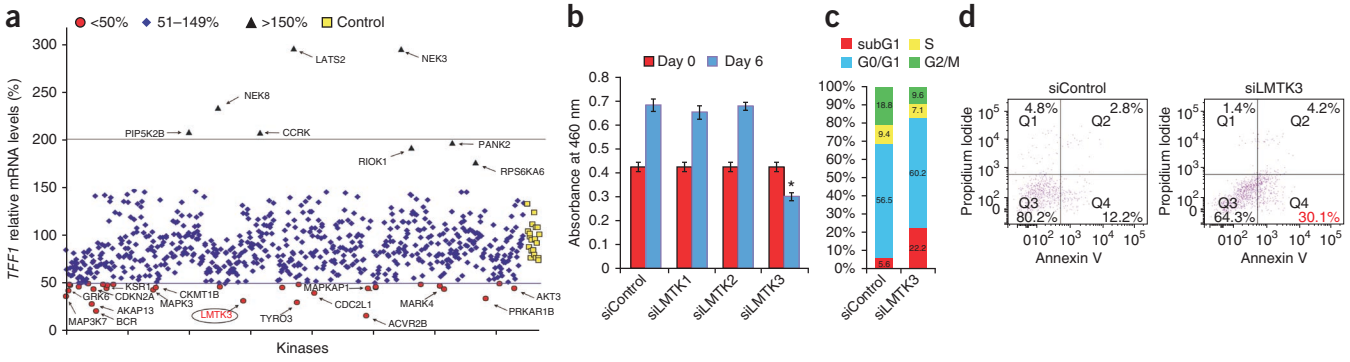

Figure 1 High-throughput siRNA screen identifies kinases modulating ERα transcriptional activity. (**a**) Relative mRNA levels of *TFF1* expression for each kinase in MCF-7 cells transfected with a pool of two different siRNAs per gene followed by E2 treatment (100% = *TFF1* mRNA expression after E2 treatment alone). $P < 0.05$. (**b**) Effects of LMTK3 silencing on MCF-7 cell proliferation. $^*P < 0.001$ (Student's *t* test). siControl, non targeting siRNA. (**c**) The proportion of MCF-7 cells in subG$_1$ phase (apoptosis) after treatment with LMTK3 siRNA or with a control siRNA. (**d**) Representative cytograms and quantification of apoptosis upon LMTK3 silencing or treatment with a control siRNA. Error bars represent s.d. of two separate experiments in triplicate.

regulate ERα, only LMTK3 has been subject to recent Darwinian positive selection compared to its chimpanzee ortholog (**Supplementary Table 2**). Further, LMTK3 silencing consistently inhibited the expression of estrogen-regulated genes potently (**Supplementary Fig. 2a** and **Supplementary Fig. 3**), whereas transfection of LMTK3 in ERα-positive breast cancer cell lines (MCF-7 and ZR-75-1) resulted in opposite effects, as measured by quantitative RT-PCR analysis. (**Supplementary Fig. 2b**).

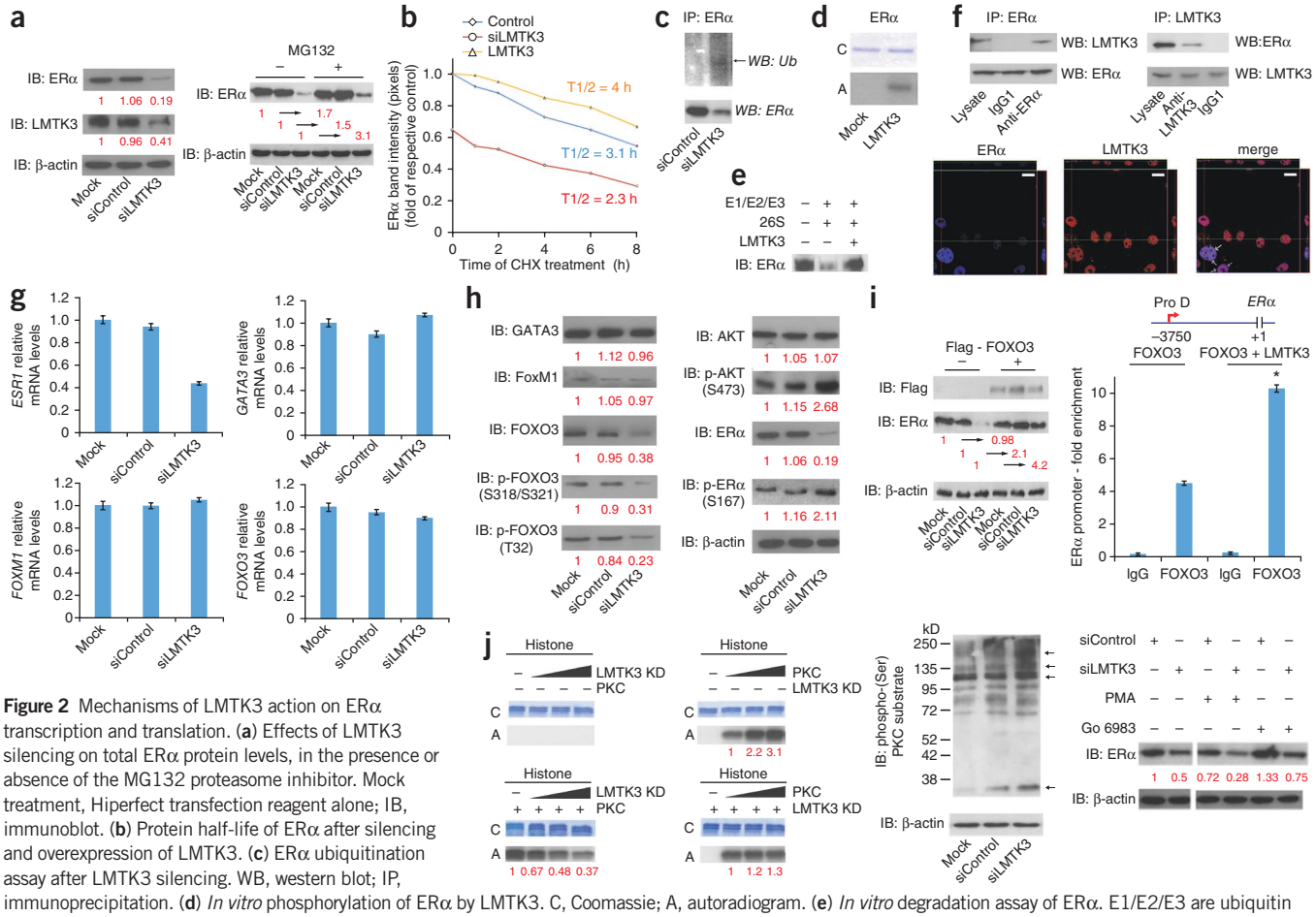

Figure 2 Mechanisms of LMTK3 action on ERα transcription and translation. (**a**) Effects of LMTK3 silencing on total ERα protein levels, in the presence or absence of the MG132 proteasome inhibitor. Mock treatment, Hiperfect transfection reagent alone; IB, immunoblot. (**b**) Protein half-life of ERα after silencing and overexpression of LMTK3. (**c**) ERα ubiquitination assay after LMTK3 silencing. WB, western blot; IP, immunoprecipitation. (**d**) *In vitro* phosphorylation of ERα by LMTK3. C, Coomassie; A, autoradiogram. (**e**) *In vitro* degradation assay of ERα. E1/E2/E3 are ubiquitin ligases; 26S is the proteasome fraction. (**f**) Coimmunoprecipitation and coimmunofluorescence of ERα and LMTK3. Scale bar, 50 μm. (**g**) Gene expression of *ESR1*, *GATA3*, *FOXO3* and *FOXM1* after LMTK3 silencing. (**h**) Effects of LMTK3 silencing on FOXO3, p-FOXO3 Ser318/Ser321, p-FOXO3 Thr32, p-AKT Ser473 and p-ERα Ser167 protein levels. In red is the fold change compared to the mock-treated samples relative to actin loading control. (**i**) Left, effects of FOXO3 overexpression on ERα protein levels. Right, effects of LMTK3 overexpression on FOXO3 binding to the *ESR1* promoter. (**j**) Left, *in vitro* kinase assays examining the effect of LMTK3 phosphorylation on the catalytic activity of PKC. Middle, effects of LMTK3 silencing on the ability of PKC to phosphorylate its substrates *in vivo*. Right, effects of LMTK3 silencing on ERα protein amounts in the presence of a PKC activator (PMA) or a PKC inhibitor (Go 6983). Error bars represent s.d. of two independent experiments in triplicate.

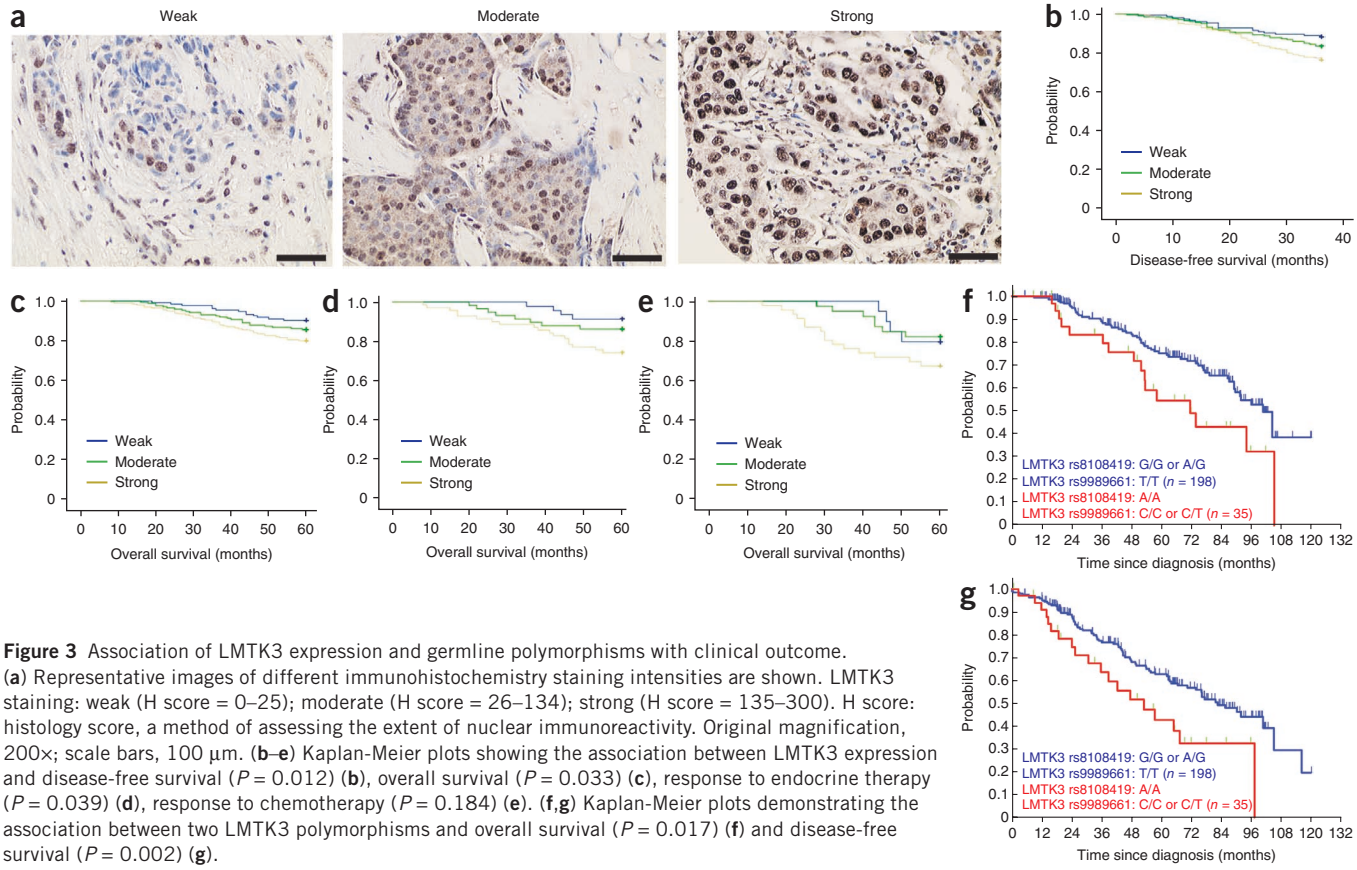

Figure 3 Association of LMTK3 expression and germline polymorphisms with clinical outcome. (**a**) Representative images of different immunohistochemistry staining intensities are shown. LMTK3 staining: weak (H score = 0–25); moderate (H score = 26–134); strong (H score = 135–300). H score: histology score, a method of assessing the extent of nuclear immunoreactivity. Original magnification, 200×; scale bars, 100 µm. (**b**–**e**) Kaplan-Meier plots showing the association between LMTK3 expression and disease-free survival ($P = 0.012$) (**b**), overall survival ($P = 0.033$) (**c**), response to endocrine therapy ($P = 0.039$) (**d**), response to chemotherapy ($P = 0.184$) (**e**). (**f**,**g**) Kaplan-Meier plots demonstrating the association between two LMTK3 polymorphisms and overall survival ($P = 0.017$) (**f**) and disease-free survival ($P = 0.002$) (**g**).

LMTKs are a family of serine-threonine-tyrosine kinases[19–21]. A function has not been ascribed to LMTK3, although screens have suggested a putative role in the β-catenin pathway[22] and leukemic cell survival[23]. We found that only LMTK3 isoform knockdown, and not LMTK1/2 isoform knockdown, inhibited the activity of an estrogen-regulated luciferase reporter, whereas LMTK3 did not alter *GAPDH* or *MCL1* expression (**Supplementary Figs. 2c**–**e**, **4** and **5**). In addition, knockdown of LMTK3, but not LMTK1 or LMTK2, inhibited MCF-7 human breast adenocarcinoma E2-dependent cell growth (**Fig. 1b**), accompanied by accumulation of cells in the sub-G1 phase (**Fig. 1c**,**d**). We obtained similar results in other ERα$^+$ cell lines and saw no effects in ERα$^-$ cells (**Supplementary Fig. 6**). Taken together, these data indicate that LMTK3 is a regulator of ERα activity.

To establish the mechanisms of LMTK3 regulation of ERα in MCF-7 cells, we next examined ERα expression. ERα protein amounts were reduced 80% by LMTK3 knockdown (**Fig. 2a**). ERα amounts were higher in the presence versus absence of proteasome inhibitors (**Fig. 2a**), and the ERα half-life was reduced after LMTK3 knockdown, whereas LMTK3 overexpression stabilized ERα (**Fig. 2b**). There was an increase in ERα ubiquitination after LMTK3 knockdown (**Fig. 2c**). Moreover, phosphorylation of ERα by LMTK3 (as suggested by data in **Fig. 2d**) protected ERα from *in vitro* proteasomal degradation (**Fig. 2e**), and LMTK3 and ERα were able to interact *in vivo* (**Fig. 2f**). Together, these data indicate that LMTK3 regulates ERα by phosphorylating and protecting it from proteasomal degradation. Next, we wished to understand the contribution of LMTK3 to *ESR1* transcription. We observed that LMTK3 knockdown reduced expression of *ESR1* mRNA (**Fig. 2g**). ERα expression is regulated by GATA binding protein 3 (encoded by *GATA3* (ref. 24)), FOXO3 (refs. 25–27) and forkhead box M1 (encoded by *FOXM1* (ref. 28)), as well as by ERα regulating its own expression[29]. LMTK3-targeting siRNA (LMTK3 siRNA) did not affect mRNA levels of these genes (*FOXO3*, *GATA3* and *FOXM1*) (**Fig. 2g**); however FOXO3 protein abundance was reduced 70%, and FOXO3 phosphorylation was reduced relative to total FOXO3 abundance (**Fig. 2h**). Overexpression of FOXO3 partially rescued the LMTK3 siRNA–mediated decrease in ERα (**Fig. 2i**), whereas chromatin immunoprecipitation confirmed that overexpression of LMTK3 increased binding of FOXO3 to the *ESR1* promoter (**Fig. 2i**). As it has already been described that AKT phosphorylates and inhibits FOXO3 by promoting its degradation[30], we examined the effects of LMTK3 silencing on AKT. We found no changes in total AKT abundance, but we did see an increase in phosphorylated cytoplasmic AKT (on Ser473), suggesting that LMTK3 siRNA–induced FOXO3 downregulation is regulated via AKT (**Fig. 2h**). Notably, we observed an increased phosphorylation of ERα at Ser167, despite decreased total ERα amounts, as a result of activated AKT, as previously described[8] (**Fig. 2h**). As protein kinase C (PKC) activity has been implicated in ERα protein degradation[31] and in decreased *ESR1* transcription via activation of AKT and inhibition of FOXO3 (ref. 32), we examined the effects of LMTK3 on PKC. *In vitro* kinase assays indicated that LMTK3 inhibits the ability of PKC to phosphorylate histone (**Fig. 2j**), whereas the use of a specific phospho-serine PKC substrate antibody showed that LMTK3 silencing increased the ability of PKC to phosphorylate a number of substrates (**Fig. 2j**). In addition, inhibition of PKC, using the Go 6983 inhibitor[33], partly rescued the downregulation of ERα protein induced by LMTK3 silencing, whereas concurrent treatment with a PKC activator (PMA)[34] and LMTK3 siRNA resulted in further degradation of

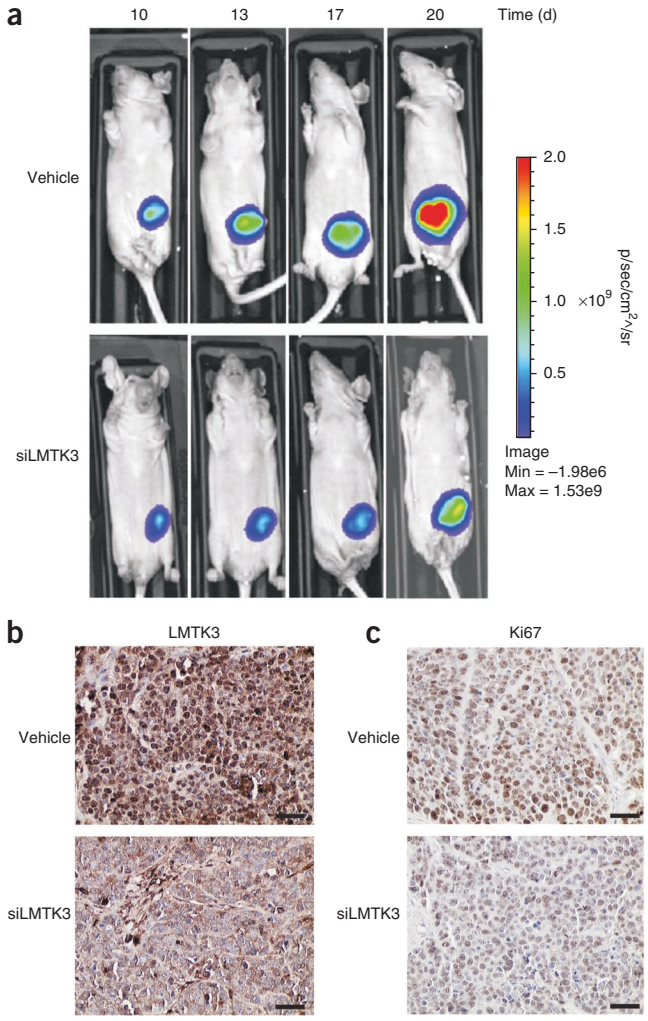

Figure 4 Tumor growth inhibition by *in vivo* LMTK3 siRNA in an orthotopic mouse model. (**a**) Bioluminescent images of a representative mouse for each group ($n = 8$) ($P < 0.01$). Tumor volume (in mm^3) was used for statistical analysis. Vehicle, PBS alone. (**b**,**c**) Histological analysis of LMTK3 (**b**) and Ki67 (**c**) expression in representative tumor tissue sections of LMTK3 siRNA–treated versus vehicle-treated tumors. Original magnification, 200×. Scale bars, 100 μm.

ERα (**Fig. 2j**). These data further imply that the effects of LMTK3 on ERα are both directly (*ESR1*) and indirectly (ERα protein) mediated via PKC signaling.

Our findings indicate that LMTK3 is key in regulating ERα activity. To confirm these data in primary breast cancer, we used immunohistochemistry to determine LMTK3 abundance (**Fig. 3a** and **Supplementary Fig. 7**) in 613 breast cancer samples[35]. High nuclear LMTK3 expression was associated with a significantly shorter disease-free survival time ($P = 0.01$) and overall survival time ($P = 0.03$) (**Fig. 3b**,**c**). LMTK3 abundance was also predictive of response to endocrine therapy ($P = 0.04$) (**Fig. 3d**) but did not predict response to adjuvant chemotherapy ($P = 0.18$) (**Fig. 3e**). To further investigate the potential involvement of LMTK3 in the development of tamoxifen resistance, we analyzed the effects of LMTK3 silencing in tamoxifen-resistant cell lines (BT-474, MLET5 and LCC9)[36–38]. Tamoxifen alone slightly affected baseline levels of cell growth, whereas addition of LMTK3 siRNA increased the growth inhibitory effects of tamoxifen, and the expected elevated levels of phosphorylated ERα and its major oncogenic co-activator amplified in breast cancer 1 (AIB1) (ref. 39) were decreased, (**Supplementary Fig. 8**). In addition, LMTK3 was also essential for E2-induced growth, as silencing of LMTK3 impeded cell proliferation in the presence of E2 (**Supplementary Fig. 9**).

The significant associations of LMTK3 expression with clinical outcome led us to test whether methylation might have a role in transcription or translation of LMTK3. We found only five out of 227 subjects[12] (**Supplementary Table 3**) with a methylated *LMTK3* gene, suggesting that methylation is not a prevalent mechanism in the control of *LMTK3* expression in this context. In this cohort, we then examined polymorphisms that could underlie the differential levels of *LMTK3* in people with cancer. We found that two intronic polymorphisms (see Online Methods) were independently associated with disease-free survival and overall survival, suggesting functionally relevant polymorphisms. Individuals harboring the *LMTK3* rs8108419 GG or AG and the *LMTK3* rs9989661 TT alleles were at a lower risk of developing tumor recurrence, which is our reference with a relative risk = 1 compared to patients carrying the *LMTK3* rs8108419 AA and *LMTK3* rs9989661 CT or CC alleles who have an increased risk (relative risk = 2.44; confidence interval: 1.40–4.25) ($P = 0.002$; **Supplementary Table 4**). Overall survival was associated with combined analyses of risk of these two polymorphisms ($P = 0.017$; **Fig. 3f**,**g** and **Supplementary Table 4**). In multivariate analyses, *LMTK3* polymorphisms were an independent prognostic factor for both disease-free survival and overall survival (**Supplementary Table 4**). Next, to investigate the effects of LMTK3 knockdown on breast tumor xenograft growth, we injected naked LMTK3 siRNA, diluted in PBS, into pre-established human MCF-7 breast carcinoma tumors grown in nude mice. *In vivo* bioluminescence imaging of the xenografted tumors showed that loss of LMTK3 protein expression, observed by immunohistochemistry, leads to a significant decrease in tumor growth (**Fig. 4** and **Supplementary Table 5**, $P = 0.024$).

The majority of human breast tumors express ERα, and individuals with $ERα^+$ disease usually respond to endocrine therapies. Endocrine resistance is a major problem, highlighting a need for understanding the mechanisms of ERα action and the development of new therapeutic agents. By performing a kinome siRNA screen to identify new proteins modulating ERα transcriptional activity, combined with evolutionary and mechanistic analyses, we have established a role for LMTK3 in regulating ERα in breast cancer. We propose a model where LMTK3 regulates the stability and activity of ERα at the mRNA level, via down-regulation of PKC catalytic activity resulting in less phosphorylated AKT (Ser473) that stabilizes FOXO3, which in turn leads to increased ERα transcriptional activity, and at the protein level, directly by phosphorylating ERα and protecting it from proteasomal degradation (**Supplementary Fig. 10**). Notably, LMTK3 expression was down-regulated by E2 and upregulated in response to tamoxifen, revealing a feedback loop between LMTK3 and ERα (**Supplementary Fig. 11**).

The demonstration that expression of and polymorphisms in *LMTK3* are associated with clinical outcome and response to endocrine therapy in breast cancer, in combination with our *in vivo* studies, suggests clinical and translational relevance. Although presumably all proteins must have been positively selected for their biochemical functions at some time in the past, very few show evidence of such adaptive evolution[40]. It is relevant that LMTK1 and LMTK2 are not positively selected for between humans and chimpanzees; rather, they are well conserved. Positive selection has been operational on human LMTK3 (in a region containing no recognized conserved kinase domains (data not shown), which may have altered

the characteristics of human versus chimpanzee LMTK3. Although the selective pressure that drove this adaptive event is at present unclear, an evolutionary tradeoff may have led to increased human susceptibility to breast cancer. Most humans we examined had the 'protective' TT allele, whereas the less-susceptible nonhuman primates lacked the protective TT allele as a result of selective pressure to counter possible deleterious effects of sequence changes to human LMTK3 (**Supplementary Table 6**). Further investigation of chimpanzee LMTK3 may yield insights into the natural history of breast cancer in humans versus chimpanzees. Together, our data reveal LMTK3 as a potential biomarker of response to endocrine therapy in breast cancer and highlight its potential as a therapeutic target.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

G.G. and J.S. conceived of the study, initiated, designed, supervised and conducted most of the experiments and wrote the manuscript. W.M., S.A., H.-J.L., C.T.-S. and R.C.C. contributed to manuscript editing. G.G. and J.J. performed the kinome screening. A.F., B.A.S., A.P., L.C. and H.Z. performed *in vitro* experiments (including proliferation assays, quantitative RT-PCR and FACS). A.F. and J.S. performed the immunohistochemistry scoring. D.Y., W.Z. and H.J.L. generated the single-nucleotide polymorphism data. W.M. produced all the evolutionary data. A.R.G. and I.O.E. performed the statistical analysis of the clinical data. All authors discussed the results, conceived further experiments, commented on the manuscript and approved the final submitted version.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

High-throughput siRNA screening. The human kinase siRNA Set Version 3.0 library (Qiagen) targeting 691 kinases and kinase-related genes was used. The library was supplied in a 96-well format and contained a pool of two individual siRNAs per well, targeting two different sequences for each gene. MCF-7 cells (American Type Culture Collection) were maintained in phenol red–free medium with 10% charcoal-stripped serum (DSS) 48 h before experimentation. Cells were plated in 24-well plates and transfected with siRNA (Qiagen) (final concentration 20 nM) using the Human Kinase siRNA Set Version 3.0 library and Hiperfect reagent according to the manufacturer's instructions (Qiagen). At 48 h after transfection, cells were treated with vehicle (ethanol) or E2 (10 nM) for 24 h, and cells were collected following RNA extraction and cDNA synthesis. Quantitative RT-PCR analysis to examine the expression of *TFF1* and *GAPDH* (endogenous control) was performed for each well (kinase gene). Next, the *TFF1* gene expression after silencing each kinase individually was calculated in relation to *GAPDH* expression; screening was performed in duplicate.

Evolutionary analysis. Positive selection on the protein-coding regions of *LMTK3* was detected by use of molecular evolution algorithms that characterize the relative proportion of nonsynonymous (replacement) nucleotide substitutions as compared to synonymous (silent) nucleotide substitutions in the kinase coding sequences. (The *LMTK3* rs8108419 GG or AG and the *LMTK3* rs9989661 TT alleles were not examined in this manner, as these regions are exclusively intronic.) All kinases shown in our screen to modulate ERα, as well as the isoforms LMTK1 and LMTK2, were examined for evidence of sequence-level positive selection between human and chimpanzee orthologs using Li93 software (a kind gift from W. Hsiung-Li). Both whole coding sequence and subsection sliding windows were examined. Only *LMTK3* showed evidence of positive selection ($P < 0.005$).

Candidate polymorphisms and genotyping. Candidate *LMTK3* polymorphisms were chosen with the assistance of the Ensembl program (http://www.ensembl.org/) using two main criteria. First, the polymorphism had to have some degree of likelihood of altering the function of the gene in a biologically relevant manner. The rs8108419 polymorphism is located in intron 2 of the *LMTK3* gene, whereas the rs9989661 polymorphism is located in intron 15 of the *LMTK3* gene. Intron polymorphisms can change gene transcription levels by alternative splicing or by affecting binding of a transcription factor. Second, the frequency of the polymorphism had to be sufficient that its impact in clinical outcome would be meaningful on a population level (above 10% allele frequency). Genomic DNA was extracted from microdissected tissue specimens using the QIAamp kit (Qiagen). *LMTK3* polymorphisms (rs8108419 and rs9989661) were tested by the PCR restriction fragment length polymorphism (PCR-RFLP) technique. Briefly, forward primer 5′-ATTCCACCACTCCCTCCAG-3′ and reverse primer 5′-GACCCTGCAGTGCCTCAC-3′ for rs8108419 and forward primer 5′-GGGCCTTCCCAAGTGGTT-3′ and reverse primer 5′-ATCCAAGCCTGGGGTGAG-3′ for rs9989661 were used for PCR amplification; PCR products were digested by the restriction enzyme BsrD1 (rs8108419) or Btsc1 (rs9989661), and alleles were separated on 4% NuSieve ethidium bromide–stained agarose gel (Lonza Rockland). Samples were obtained with approval from the Riverside Ethics Committee with appropriate informed consent from the subjects.

***In vivo* tumorigenicity assay in nude mice bearing orthotopic breast cancer xenografts.** Bioluminescent MCF-7 breast cancer cell lines (PRECOS) were injected into the mammary fat pad of nude mice (PRECOS). When tumors reached an approximate volume of 100–200 mm^3 (day 15), mice were randomly assigned to different groups ($n = 8$, each group) to receive intratumoral injections of 10 μg *in vivo*–modified LMTK3 siRNA or control siRNA (Qiagen). Three intratumoral injections were repeated every 3 d (50 μl volume per injection), and mice were killed 3 d after the last injection. Tumor growth was monitored with caliper measurements, and bioluminescent imaging was performed 24 h before dosing and 72 h after dosing. After the mice were killed, primary tumors were excised, weighed and formalin fixed. Samples were paraffin embedded, cut at 3 μm and H&E stained for histological evaluation of target proteins expression. This study was conducted under the UK Home Office Licence number PPL 40/2962.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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Feature Article

MicroRNAs and RNA-binding proteins

Jimmy Jacob[1], Justin Stebbing[1], Adam Frampton[2], Loredana Pellegrino[1] and Leandro Castellano[1]

[1]*Department of Oncology, Imperial College, London, UK and* [2]*HPB Surgical Unit, Department of Surgery and Cancer, Imperial College London, UK*

Introduction

MicroRNAs (miRNA) and RNA-binding proteins (RBPs) are important post-transcriptional gene regulators. RBPs influence the structure and interactions of the RNAs and play critical roles in their biogenesis, stability, function, transport and cellular localisation. It is becoming apparent that miRNA activity is not necessarily always determined by its expression in the cell; miRNA activity can be affected by RBPs. Recent studies have introduced a paradigm shift in our understanding of the miRNA biogenesis pathway, which was previously believed to be universal to all miRNAs. It has now been proved that maturation steps of individual miRNAs could be specific. RBPs like KSRP, Lin-28 and many more have been proved important for the biogenesis of miRNAs and this regulation is crucial, as alteration of miRNA expression has been linked to several cancers. Here we review the recent progress in our understanding of the miRNA biogenesis pathways regulated by RBPs which could be potential therapeutic targets for different cancers.

MicroRNA biogenesis

miRNAs are endogenous non-coding RNAs of approximately 22 nucleotides which, as post-transcriptional modulators of gene expression, mechanistically act by base-pair interaction with the 3′ UTR (prime untranslated region) of the messenger RNA (mRNA), thereby inducing post-transcriptional repression [1]. In other circumstances, when the miRNAs interact partially with the 3′ UTR of their target mRNAs, they induce their translational repression [2,3]. miRNAs are regulators of gene expression that control many biological processes in development, differentiation, growth and metabolism [4].

miRNAs were first discovered in nematodes in 1993 [5]. A single miRNA can bind to and regulate many different mRNA targets and, conversely, several different miRNAs can bind to and cooperatively control a single mRNA target [6]. Analogous to transcription factors, miRNAs regulate mRNAs in a combinatorial fashion and single miRNAs can repress the translation of many mRNAs [7]. Recently, Eiring *et al*. [8] showed that *miR-328* (miRNA-328) has a second function, acting as a decoy by binding to hnRNP E2 (heterogeneous nuclear ribonucleoprotein E2) and lifting its translational repression of an mRNA involved in myeloid cell differentiation.

miRNA genes are evolutionarily conserved and may be located either within the introns or exons of protein-coding genes (70%) or in intergenic areas (30%) [9]. Transcription of miRNAs is typically performed by RNA polymerase II, then the transcripts are capped and polyadenylated [10]. A transcript may encode clusters of distinct miRNAs, or it may encode an miRNA and a protein. The resulting primary or pri-miRNA transcript extends both 5′ and 3′ from the miRNA sequence, and two sequential processing reactions trim the transcript into the mature miRNA. In the first processing step, which occurs in the nucleus, the pri-miRNA is cleaved into pre-miRNA (an approximately 70-nucleotide hairpin stem loop intermediate) by a microprocessor complex that consists of Drosha, an RNase III enzyme, and DGCR8, a double-stranded RNA-binding domain (dsRBD) protein [7]. The microprocessor complex also contains a variety of cofactors including the DEAD box (amino acid sequence D-E-A-D asp-glu-ala-asp) RNA helicases p68 (DDX5) and p72 (DDX17), as well as heterogeneous nuclear ribonucleoproteins (hnRNPs) [11,12]. These auxiliary factors may function to promote the fidelity, specificity or activity of Drosha cleavage. Surprisingly, Drosha-mediated processing of pri-miRNAs into pre-miRNAs is not obligatory. A few intron-derived miRNAs called mirtrons are directly released from their host transcripts after splicing as pre-miRNA, so bypassing the Drosha cleavage [13,14]. After its formation, the pre-miRNA is actively transported from the nucleus to the cytoplasm by the Ran-GTP-dependent transporter Exportin 5 (Exp 5) [15,16]. Drosha cleavage occurs co-transcriptionally, before splicing of the host RNA [17], and generates a product with a two-nucleotide 3′ overhang, characteristic of RNase III-mediated cleavage. The overhang is recognised by Exportin-5, which transports the pre-miRNA into the cytoplasm via a Ran-GTP-dependent mechanism [18,19]. In the cytoplasm, the pre-miRNA is further 'diced' into an approximately 22-nucleotide miRNA duplex by Dicer, another RNase III enzyme, in collaboration with the dsRBD proteins TRBP/PACT [20–22]. The two miRNA strands are then separated and one of the strands associates with an Argonaute (AGO) protein within the RNA-induced silencing complex (RISC or miRISC) where it acts as a guide to repress target messages. The miRNA guides RISC to complementary sites within the target mRNAs to mediate repression of that target message.

RNA-binding proteins

RNA-binding proteins have a major role in every aspect of RNA biology, from transcription, pre-mRNA splicing and polyadenylation to RNA modification, transport, localisation, translation and turnover [23]. RNAs in cells are associated with RBPs to form ribonucleoprotein (RNP) complexes. Post-translational modification of RBPs generates additional layers of complexity, as it can modify the RNA binding, function and localisation of the RNP. Three types of modification have been described for RBPs: phosphorylation, arginine methylation and small ubiquitin-like modification (SUMO). The complement of RBPs present at a particular locale where the RNA is transcribed, or changes in the post-translational modifications of these proteins, would affect the resulting RNP complex, modulating its downstream functional activity [24]. Heterogeneous nuclear ribonucleoproteins (hnRNPs) are complexes of RNA and protein present in the nucleus during gene transcription and subsequent post-transcriptional modification of the newly synthesised RNA (pre-mRNA). The discovery of the heterogeneous nuclear ribonucleoproteins (hnRNP), and other pre-mRNA/mRNA-binding proteins, led to the identification of the first amino acid motifs and functional domains that confer binding to RNA [25]. RBPs contain one or more often multiple RNA-binding domains. Some well-characterised RNA-binding domains (RBD, also known as RNP domain and RNA recognition motif, RRM) include the following: K-homology (KH) domain (type I and type II), RGG (Arg-Gly-Gly) box, Sm domain, DEAD/DEAH box, zinc finger (ZnF), double-stranded RNA-binding domain (dsRBD), cold-shock domain, Pumilio/FBF (PUF or Pum-HD) domain and the Piwi/Argonaute/Zwille (PAZ) domain [24]. Many RBPs, like hnRNPs, have different domains and they tend to shuttle between nucleus and cytoplasm having roles in different cellular processes. Many signalling pathways operate by modifying the activity of specific RBPs, in which RNA binding motifs are combined with other conserved domains, such as protein–protein interaction domains and consensus phosphorylation motifs [26].

MicroRNA regulation by RNA-binding proteins

The regulation of microRNA expression is at three different levels: pre-transcriptional, transcriptional and post-transcriptional [27]. Most of the miRNA regulation by RNA-binding proteins (RBPs) is at the post-transcriptional level.

Many factors affect the biogenesis of the miRNAs at the pre-transcriptional level. Besides the gain or loss of miRNA gene copy number, by amplification, translocation or deletion, research indicates that their expression is epigenetically modulated, through histone deacetylation and hypermethylation of miRNA promoters [27]. Maturation of miRNAs requires coordinated processing mechanisms by Drosha and Dicer that post-transcriptionally generate mature miRNAs from pri-miRNA transcripts. *miR-18a* is processed from a polycistronic *pri-miR-17-92* transcript, which harbours five additional miRNAs. hnRNP A1, an mRNA splicing regulator, exclusively stimulates maturation of *miR-18a* from the *miR-17-92* cluster. Pri-miR-18a, but not other members of the cluster, contains two regions of similarity to the consensus hnRNP A1-binding site, UAGGGA/U, within its terminal loop and stem [28,29]. Direct and specific interaction of hnRNP A1 with the terminal loop and stem of the pri-miR-18a hairpin induces a structural rearrangement of the hairpin to generate a more favourable Drosha/DGCR8 binding and cleavage site. Thus, hnRNP A1 acts as a chaperone for recognition and cropping of specific pri-miRNAs by Drosha/DGCR8. Interestingly, approximately 14% of human miRNAs contain highly conserved loop sequences, suggesting that processing regulation by hnRNPs and other nuclear RBPs might extend well beyond miR-18a [29]. In addition to the general miRNA machinery, different studies have linked specific miRNAs to cancer. Oncogenic miRNA gene *miR-17-92* is the most highly expressed cluster in cancers. This is a cluster of six miRNAs located on 13q31.3, a locus that is frequently amplified in lymphomas [30]. The *miR-17-92* cluster is highly expressed in a variety of solid malignancies, including cancers of the colon, lung, pancreas, prostate and stomach, as well as lymphomas. The polycistronic miRNA cluster produces a single primary transcript yielding the six mature miRNAs: *miR-17*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b* and *miR-92a*. The sequences of the mature miRNAs are highly conserved in all vertebrates. The human miR-17-92 cluster is located in the third intron of the primary transcript C13orf25. Despite the high conservation of miRNA sequences, the exonic sequences of C13orf25 are not conserved across species, indicating that it is most likely that the only function of the transcript is the production of miRNAs [31]. It has been shown that c-MYC directly induces the transcription of the *miR-17-92* cluster during both B cell proliferation and oestrogen receptor (ESR1) induction in breast cells [32,33]. Recent studies dissected the miR-17-92 cluster to its individual miRNA components and identified their relative contributions to oncogenic transformation in mouse model systems [34,35]. They identified miR-19 as the potent oncogene of the miR-17-92 cluster by inhibiting the expression of the tumour suppressor PTEN; miR-19 promotes cellular growth and cancer.

Many miRNAs are regulated at the Dicer processing step. *Pre-miR-138* is expressed ubiquitously but its mature form is restricted just to certain cell types, indicating tissue-specific Dicer processing of this miRNA [36]. Recently, it has been demonstrated that

Lin-28 is a stem-cell-specific regulator of *let-7* post-transcriptional maturation that controls both Drosha and Dicer processing. In the nucleus, Lin-28 inhibits microprocessor-mediated cleavage of *pri-let-7* [37], by competing with Drosha [38,39]. In the cytoplasm, Lin-28 is able to inhibit Dicer-mediated cleavage of the *pre-let-7* family member [40] by recruiting a terminal uridylyl transferase (TUTase) to the precursor. The uridylated *pre-let-7* (up-let-7) cannot be processed by Dicer and is degraded by an unidentified nuclease [41]. The KH-type splicing regulator protein (KSRP, also known as KHSRP) recognises G-rich regions, including a GGG triplet, present within the terminal loops of a subset of pri-miRNAs, to promote Drosha/DGCR8-mediated processing [42]. Upon binding, KSRP can optimise the positioning and recruitment of the miRNA precursor-processing complexes through protein–protein interactions. KSRP is associated with the terminal loop of the target miRNA precursors during nucleocytoplasmic transit and also promotes the processing of the target pre-miRNAs by Dicer in the cytoplasm. This further suggests that specific recognition of the terminal loop by RBPs is an important means to extend regulation of miRNA processing down to the level of individual miRNAs. Binding of RBPs to specific sequences present in the terminal loop regions of miRNA precursors potentially either competes for binding or promotes recruitment of processing factors. Changes of expression, RNA-binding activity, interacting protein partners and subcellular localisation of RBPs in response to extracellular signals might serve to regulate biogenesis of individual miRNAs.

It is well established that RBPs can modulate the function of miRNAs in different ways either through the biogenesis of miRNAs or by directly interacting with the miRNA binding sites; for example proteins like Deadend 1 or HuR (Hu antigen R, also known as ELAV1) can compete with miRNA binding sites on mRNAs and modulate miRNA function [43]. Recently, it was discovered that *miR-328* has a second function, acting as a decoy by binding to hnRNP E2 and lifting its translational repression of an mRNA involved in myeloid cell differentiation [8]. These findings are intriguing because an miRNA-mediated regulatory function associated with RBPs has not been reported before. Moreover, the general view of miRNA functions entails a model in which miRNAs serve as guides for Ago protein complexes, the actual mediators of post-transcriptional gene silencing. Eiring *et al*. demonstrated that *miR-328* can act independently of Ago proteins by interacting directly with hnRNP E2 and the discovery of this new function for miRNAs raises a number of fascinating questions. These findings not only offer new insights into the dual role of *miR-328* but also suggest a novel function for miRNAs as direct inhibitors of protein activity, thereby offering a paradigm shift for miRNA-mediated gene regulation. It is likely that the dual role (decay/decoy) activity of miRNAs is not limited to *miR-328* but can be extended to other miRNAs containing nucleotide sequences resembling the consensus RNA-binding sites for RBPs that are involved in cancer as well as non-cancer-related diseases. It is clear that interplay between miRNAs and RBPs exists, which is important for proper function of processes involved in differentiation, cell cycle, stress and cell survival. As miRNAs play a very prominent role in cancer and RBPs regulate miRNA biogenesis and activity, it shows miRNA–RBP interplay is important for the regulation of cancerous pathways.

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Correspondence to: Jimmy Jacob
School of Medicine
Imperial College London
5th Floor, MRC Cyclotron Building
Hammersmith Hospital Campus
Du Cane Road, London W12 0NN, UK
(email: j.jacob@imperial.ac.uk)

EXPERT REVIEWS

Integrated analysis of miRNA and mRNA profiles enables target acquisition in human cancers

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Adam E Frampton*[1], Jonathan Krell[2], Loredana Pellegrino[2], Laura Roca-Alonso[2], Long R Jiao[1], Justin Stebbing[3], Leandro Castellano[2] and Jimmy Jacob[2]

[1]*HPB Surgical Unit, Department of Surgery and Cancer, Imperial College, Hammersmith Hospital, Du Cane Road, London, W12 0HS, UK*
[2]*Division of Cancer, Department of Surgery and Cancer, 5th Floor, MRC Cyclotron Building, Imperial College, Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK*
[3]*Department of Oncology, Imperial College Healthcare NHS Trust, Charing Cross Hospital, 1st Floor, E Wing, Fulham Palace Road, London, W6 8RF, UK*
**Author for correspondence: a.frampton@imperial.ac.uk*

Evaluation of: Buffa FM, Camps C, Winchester L *et al.* miRNA-associated progression pathways and potential therapeutic targets identified by integrated mRNA and miRNA expression profiling in breast cancer. *Cancer Res.* 71(17), 5635–5645 (2011).

miRNAs play a role in post-transcriptional gene regulation by translational repression and/or mRNA degradation in a very tissue-specific manner. In order to understand the function of a miRNA, it is best to identify the genes that it regulates. Putative mRNA targets of miRNAs identified from seed sequence matches are available using computational algorithms in various web-based databases. However, these tend to have high false-positive rates and, owing to a whole-genome approach, cannot identify tissue/tumor specificity of regulation. The evaluated article presents a large amount of data analyzing global RNA expression in breast cancer and examines whether miRNAs are prognostic due to their effects on mRNA targets. This valuable and important resource of combined miRNA and mRNA expression in breast cancer and its subtypes has been summarized. Many studies have now investigated the integrated analysis of miRNA:mRNA profiles in human malignancies, the goal as always being to identify novel biomarkers and therapeutic targets for each tumor.

KEYWORDS: breast cancer • microRNA • miR-27b • miR-128a • miR-150 • miR-210 • miR-342 • mRNA • pri-miRNA • processing

Summary of methods & results

Methods

Clinical samples

A cohort of patients with early primary breast cancer (BC) who received surgery in Oxford, UK (1989–1993) was considered (n = 219, of which 207 were suitable for RNA analysis) [1]. Complete 10-year follow-up was available for all patients. Tumor subtypes included estrogen receptor negative (ER^-; n = 82), positive (ER^+; n = 90) and triple-negative (n = 37). Total RNA was extracted from whole fresh frozen tumor samples after visual assessment. No micro-dissection of tumor cells was performed, as there is evidence that signaling from the stroma has an important role in cancer progression [2].

RNA-expression profiling

Matched miRNA and mRNA profiling was performed by using Illumina human ref seq-8 and miRNA v1 arrays. Primary miRNA transcript (pri-miRNA) expression was therefore also available.

Association of miRNA expression with survival independent of clinicopathological factors & validation using other datasets

Penalized Cox regression was used to identify miRNAs that were prognostic for distant relapse-free survival (DRFS) after surgery, independent of known clinicopathological factors (i.e., age, tumor size and grade, lymph node status, ER status, chemotherapy treatment, and gene signatures of biological processes). Previously published BC cohorts with available Affymetrix U133A-B/plus2 array data were then investigated to validate the miRNAs identified in the Oxford cohort. Four additional datasets were analyzed for DRFS (n = 592) and six datasets for recurrence-free survival (n = 1050) with

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Table 1. miRNA:mRNA integrated profiling in human cancers†.

Study (year)	Tumor type	Methods/sample size	Findings	Ref.
Enerly *et al.* (2011)	Breast cancer	n = 101 (tumor subtypes: basal-like n = 15; luminal-A n = 41; luminal-n = 12; HER2 n = 17; normal breast tissue n = 10; unknown n = 6) There was substantial overlap between miRNAs differentially expressed between ER^+ and ER^- samples, and between WT and mutant *TP53* samples, therefore they also considered: TP53 WT/ER^+ n = 50 vs TP53 mutant/ER^+ n = 11, and TP53 WT/ER^- n = 12 vs TP53 mutant/ER^- n = 26	Basal-like subtype was distinguished by higher expression of the miR-17–92 family and miR-9/9*, and lower expression of the miR-29 family miR-29c was found to be the most differentially expressed between extracellular matrix classes 1 and 4. In accordance, cell-adhesion genes were anticorrelated with miR-29c expression Sixteen miRNAs were differentially expressed between *TP53* WT and mutated samples. *TP53* mutant tumors had significantly higher expression of miR-9/9* and miR-135b, and lower expression of miR-342-3p and miR-26a. Consequently, overexpression of miR-342 leads to reduced proliferation in MCF-7 cells. Interestingly, miR-34a was not differentially expressed when comparing *TP53* mutational status in this cohort Three miRNAs were significantly upregulated in *TP53* mutant/ER^+ tumors >1.2-fold change (miR-9*, -378* and -452); four miRNAs were significantly upregulated in *TP53* mutant/ER^- tumors >twofold change (miR-431*, -127–5p, -921 and -640) miR-150 had strong enrichment of the immune response GO term among its positive correlated mRNAs; high expression of miR-150 was predictive of better prognosis in ER^-/$HER2^-$ patients	[15]
Borgan *et al.* (2011)	Breast cancer	Excised breast cancer tissue (n = 10) was macrodissected, then left at room temperature and frozen in liquid nitrogen at four different time points following surgery (0.5, 1, 3 and 6 h)	Time to snap freezing (ischemia time) affected the expression of: 56 miRNAs (all increased) including miR-1224-5p, miR-1225-5p, miR-125a-3p, miR-371-5p, miR-483-5p, miR-663, miR-557 and miR-765 1788 mRNAs were up-/down-regulated mRNAs significantly increased with ischemia time included those for the transcription factors of the Jun, Fos, Maf and ATF subfamilies Deregulated miRNA-associated biological processes included 'response to stimulus', 'response to wounding' and 'stress response'. These miRNAs showed enrichment of positively or negatively correlated mRNAs Positively correlating miRNA–mRNA pairs that may be involved in the stress response included miR-663-FOSB, miR-939-JUND and miR-1228-CDKN1A	[23]
Sieuwerts *et al.* (2011)	Breast cancer CTC	Blood samples from MBC patients (n = 50) prior to start of systemic therapy and healthy donors (n = 53) RNA isolated from five CTCs per 7.5 ml of blood (clinically relevant cutoff) despite contaminating leukocytes	Identified 65 epithelial tumor cell-specific miRNAs (n = 10) and mRNAs (n = 55) Hierarchical clustering identified patients with a twofold higher rate of having both visceral and nonvisceral metastases Difficulty in correlating *ERBB2* and *ESR1* mRNA expression in CTCs of patients with known $HER2^+$ or ER^+ primary tumors, respectively	[24]
Van der Auwera *et al.* (2010)	IBC	384 miRNAs evaluated in IBC (n = 20) vs non-IBC (n = 50)	Six miRNAs upregulated in IBC: miR-335, miR-337-5p, miR-451, miR-486-3p, miR-520a-5p and miR-548d-5p Seven miRNAs downregulated in IBC: miR-15a, miR-24, miR-29a, miR-30b, miR-320, miR-342-5p and miR-342-3p Four miRNAs (miR-29a, miR-30b, miR-342-3p and miR-520a-5p) had targets significantly enriched among their correlated genes; GO terms over-represented in the enriched target gene set related to cell proliferation and signal transduction Prognostic relevance for the four miRNAs, using Cox regression on seven independent datasets, revealed significant associations between miRNA target gene expression and patient survival for miR-29a, miR-30b and miR-520a-5p, but not for miR-342-3p High AGO2 and low DICER1 in aggressive IBC compared with non-IBC samples	[25]

†Few digestive system tumors are represented.

5-FU: Fluorouracil; AGO2: Argonaute 2; ALL: Acute lymphoblastic leukemia; BC: Breast cancer; ccRCC: Clear-cell renal cell carcinoma; CTC: Circulating tumor cell; ER^-: Estrogen receptor negative; ER^+: Estrogen receptor positive; FdU: Fluoxuridine; FdUMP: Fluorodeoxyuridine monophosphate; GO: Gene ontology; HCPT: Hydroxycamptothecin; IBC: Inflammatory breast cancer; MBC: Metastatic breast cancer; MM: Multiple myeloma; MSI-H: High microsatellite instability; MSS: Microsatellite stable; TCC: Transitional cell carcinoma; TGCT: Testicular germ cell tumor; WT: Wild-type.

Table 1. miRNA:mRNA integrated profiling in human cancers† (cont.).

Study (year)	Tumor type	Methods/sample size	Findings	Ref.
Blenkiron *et al.* (2007)	Breast cancer	309 miRNAs evaluated in primary BC samples (n = 93), normal breast samples (n = 5) and BC cell lines (n = 21)	31 miRNAs associated with tumor subtype or clinicopathological factors; many upregulated in the less aggressive, grade 1, ER^+ cases; some upregulated in more aggressive grade 3/ER^- tumors (e.g., miR-150) miRNA-processing genes: low DICER1 in more aggressive basal-like, $HER2^+$ and luminal B-type tumors; high AGO2 in basal-like, $HER2^+$ and luminal B-type tumors; higher AGO2 and DROSHA, and lower DICER1 in ER^- tumors	[26]
Lionetti *et al.* (2009)	MM	723 human miRNAs evaluated in BM aspirates MM (n = 38), plasma cell leukemia (n = 2) and normal control samples (n = 3)	The results highlighted that specific patterns of miRNA expression may differentiate MMs with distinct and well-known genetic alterations. Specific signatures were found to be associated with t(4,14) or translocated *MAF* genes, and to a lesser extent with t(11,14) Identified 26 miRNAs significantly discriminating the translocation/cyclin groups that have previously been found to be involved in solid and hematologic tumors. The most extensively investigated were miR-155, miR-221 and miR-222, and the let-7 family	[27]
Fulci *et al.* (2009)	ALL	470 miRNAs evaluated in patients with ALL (n = 52; of these T-ALL n = 9 and B-ALL n = 43)	Showed a downregulation of miR-151 in patients with T-ALL, while miR-148a and miR-424 displayed higher expression This approach also highlighted six miRNAs that exhibited patterns of expression that are class specific: miR-425–5p, miR-191 and miR-128 were preferentially expressed in the *E2A/PBX1*-positive cases; miR-629 was highly expressed in cases harboring *MLL/AF4* rearrangement; while high levels of miR-146b and miR-126 were observed in the *BCR/ABL*-positive cases	[28]
Wu *et al.* (2011)	Gastric cancer	723 human miRNAs evaluated in HCPT-resistant vs HCPT-sensitive cell lines	In HCPT-resistant gastric cancer cells, 25 miRNAs may play important roles in intrinsic HCPT resistance; 12 miRNAs were downregulated and the rest were upregulated 307 genes were differentially expressed in HCPT-resistant cell lines, including apoptosis-related genes (*BAX*, *TIAL1*), cell division-related genes (*MCM2*), cell adhesion or migration-related genes (*TIMP2*, *VSNL1*) and checkpoint genes (*RAD1*) Hierarchical clustering based on the expression patterns of 25 miRNAs showed that cell groupings were generally consistent with HCPT sensitivity	[29]
Lanza *et al.* (2007)	Colorectal cancer	Matched bulk normal colonic mucosa and colorectal cancer tissue snap frozen in liquid nitrogen at time of surgery; n = 39 (tumor subtypes: MSS n = 23; and MSI-H n = 16)	No interactions identified, but mRNA/miRNA expression signatures are able to improve biomolecular classification of colorectal cancer 27 differentially expressed genes could correctly distinguish MSI-H vs MSS This molecular signature included eight miRNAs (miR-223, -155, -191, -32, -25, -92-1, -92-2 and -93-1) Members of the oncogenic miR-17-92 family (miR-17-5p, -20, -25, -92-1, -92-2 and -93-1) were significantly upregulated in MSS cancers	[30]
Li *et al.* (2011)	Colorectal cancer	Colorectal cancer tissues (n = 7) and normal colonic mucosa (n = 4)	A total of 31 downregulated miRNAs and two upregulated miRNAs; 73 upregulated mRNAs and 63 downregulated mRNAs were identified Based on the partial least-squares regression method, Li *et al.* constructed association networks including the significant miRNAs and mRNAs. The resulting miRNA:mRNA association network had 97 nodes and 155 connections between the 31 downregulated miRNAs and the 71 upregulated mRNAs Networks were also developed for the seven miRNAs (let-7 g, miR-101, miR-133a, miR-15a, miR-16, miR-29b and miR-29c) closely related to cancer and their associated mRNAs	[31]

†Few digestive system tumors are represented.
5-FU: Fluorouracil; AGO2: Argonaute 2; ALL: Acute lymphoblastic leukemia; BC: Breast cancer; ccRCC: Clear-cell renal cell carcinoma; CTC: Circulating tumor cell; ER^-: Estrogen receptor negative; ER^+: Estrogen receptor positive; FdU: Fluoxuridine; FdUMP: Fluorodeoxyuridine monophosphate; GO: Gene ontology; HCPT: Hydroxycamptothecin; IBC: Inflammatory breast cancer; MBC: Metastatic breast cancer; MM: Multiple myeloma; MSI-H: High microsatellite instability; MSS: Microsatellite stable; TCC: Transitional cell carcinoma; TGCT: Testicular germ cell tumor; WT: Wild-type.

Table 1. miRNA:mRNA integrated profiling in human cancers[†] (cont.).

Study (year)	Tumor type	Methods/sample size	Findings	Ref.
Li *et al.* (2011)	Genito-urinary cancers: bladder, testis and kidney	TCC (n = 10); TGCT (n = 7); ccRCC (n = 10) vs matched normal adjacent tissues	A total of 3181, 4321 and 4596 mRNAs were significantly differentially expressed in TGCT, TCC and ccRCC, respectively, and the numbers of miRNAs significantly deregulated in these cancers were 254, 226 and 118, respectively This huge analysis identified a number of annotated cancer-associated miRNAs such as miR-142-3p, miR-155, miR-21, miR-210, let-7c and miR-214 exhibiting consistent deregulation in TCC, TGCT and ccRCC, which may suggest their general effects on human cancer development The miR-200 family exhibited different patterns in TCC, TGCT and ccRCC, which suggested their diverse roles in the genitourinary cancers	[32]
Zhu *et al.* (2011)	Bladder cancer	Genome-wide methylation analysis and mRNA/miRNA expression profiling by second-generation sequencing in bladder tumors (n = 9) and matched normal adjacent tissues (n = 9) A further 33 bladder cancer samples were used for validation	56 miRNAs predicted to target 285 different mRNAs (including downregulation of several solute carrier family genes such as *SLC2A4*). The most significantly enriched GO terms for the identified miRNA-targets included 'protein kinase activity', 'cell cycle', 'axon guidance' and 'MAPK signaling pathways' Upregulated in bladder cancer: miR-182, miR-183, miR-10a, miR-203 and miR-224 Downregulated: miR-1, miR-143, miR-145, miR-133a, miR-133b and miR-125b 82 simultaneously differentially methylated and expressed cancer-associated genes (e.g., *ADAMTS9*, *CCND1* and *HIC1*) in bladder tumors	[33]
Liu *et al.* (2010)	Renal cell carcinoma	ccRCC tumor vs adjacent normal kidney tissue samples (n = 17)	35 miRNAs were identified as differentially expressed in ccRCC vs normal kidney: 26 downregulated and nine upregulated This method revealed many new regulations in ccRCC: loss of miR-149, miR-200c and miR-141 caused gain of function of oncogenes *KCNMA1* and *LOX*, *VEGFA* and *SEMA6A*, respectively Increased levels of miR-142-3p, miR-185, mir-34a, miR-224 and miR-21 caused loss of function of tumor-suppressor genes *LRRC2*, *PTPN13*, *SFRP1*, *ERBB4, SLC12A1* and *TCF21*, respectively In addition, there was strong anticorrelation between *VEGFA* and the miR-200 family (miR-200a*, 200b, 200c and miR-141)	[3]
Gmeiner *et al.* (2010)	Various: NCI-60 cell lines	Analysis of mRNA and miRNA expression profiles were correlated with sensitivity to polyfluoropyrimidine anti-tumor agent FdUMP, 5-FU, FdU and topoisomerase 1 agents (topotecan and irinotecan) across the NCI-60 cell line screen	Identified distinct mechanistic features of FdUMP based on its mRNA and miRNA profile compared with both alternative fluoropyrimidine drugs (i.e., 5-FU and FdU), as well as from other topoisomerase 1 poisons (i.e., irinotecan and topotecan) mRNAs affected: TGF signaling genes (*TGFBR3* and *SMAD6*) and members of the antiapoptotic Bcl2 family had significant negative correlation with sensitivity to FdUMP	[34]
Blower *et al.* (2007); Sokilde *et al.* (2011)	Various: NCI-60 cell lines	The NCI-60 panel of 60 cancer cell lines has already been profiled for mRNA and protein expression, mutational status, chromosomal aberrations and DNA copy number. miRNA expression has been examined in three studies	miRNA expression between the three NCI-60 studies was highly correlated, despite different techniques By matching drug-sensitivity profiles for the NCI-60 cells to their miRNA-expression profiles, they identified many drug–miRNA pairs, indicating that miRNAs play a role in chemoresistance	[35,36]

[†]Few digestive system tumors are represented.
5-FU: Fluorouracil; AGO2: Argonaute 2; ALL: Acute lymphoblastic leukemia; BC: Breast cancer; ccRCC: Clear-cell renal cell carcinoma; CTC: Circulating tumor cell; ER$^-$: Estrogen receptor negative; ER$^+$: Estrogen receptor positive; FdU: Fluoxuridine; FdUMP: Fluorodeoxyuridine monophosphate; GO: Gene ontology; HCPT: Hydroxycamptothecin; IBC: Inflammatory breast cancer; MBC: Metastatic breast cancer; MM: Multiple myeloma; MSI-H: High microsatellite instability; MSS: Microsatellite stable; TCC: Transitional cell carcinoma; TGCT: Testicular germ cell tumor; WT: Wild-type.

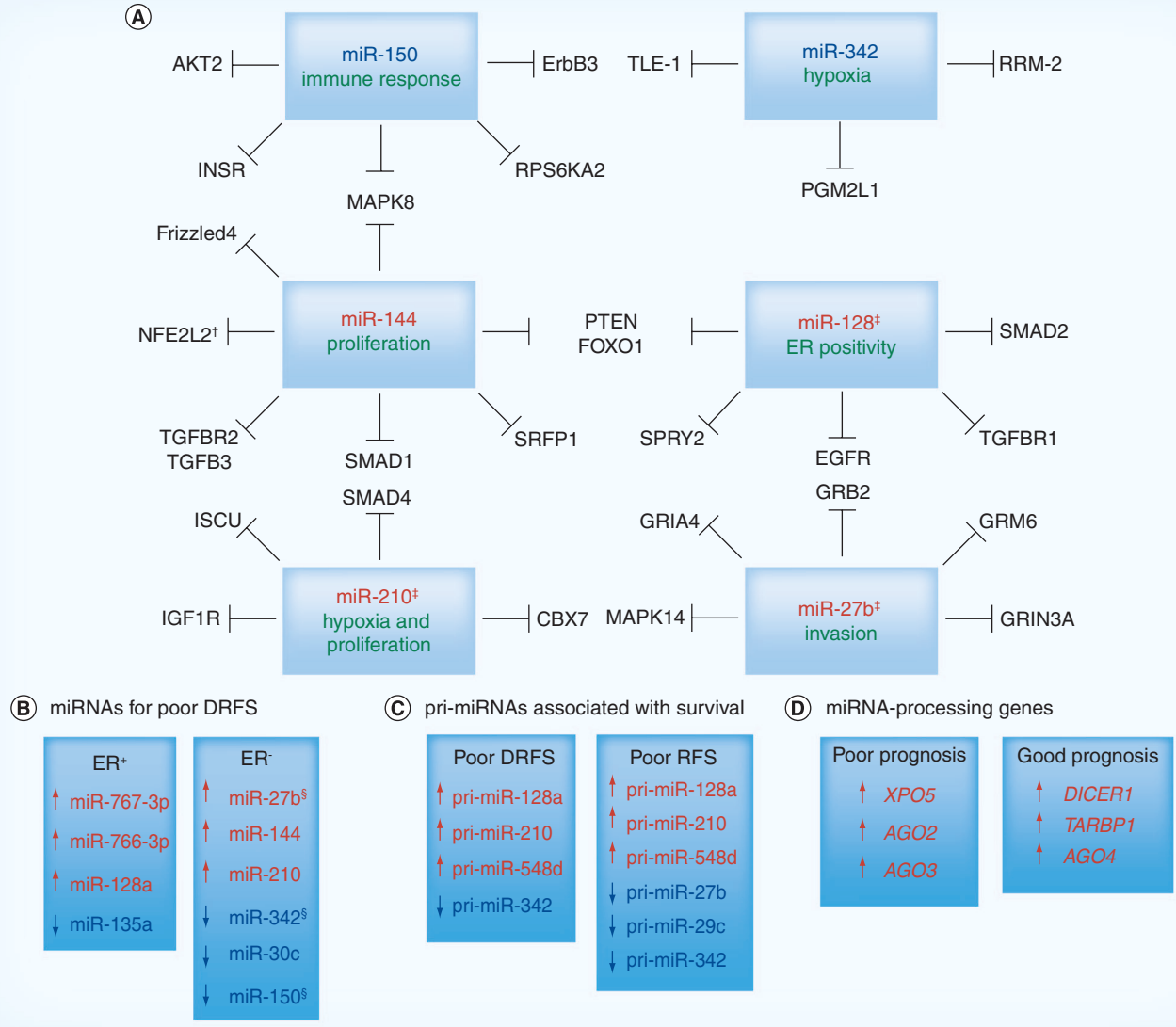

Figure 1. Pri-miRNAs, mature miRNAs and miRNA-processing genes associated with clinical outcome in breast cancer.
(A) Part of the miRNA:mRNA interaction network for prognostic miRNAs in breast cancer (green, key biological processes associated with miRNA expression; †NFE2L2 protein expression was assessed in 137 cases by immunohistochemistry and confirmed to be weak in those tumors with high miR-144; ‡downregulation of cognate targets for these miRNAs was validated in independent breast cancer datasets. **(B)** Independently prognostic miRNAs for DRFS in estrogen receptor-positive tamoxifen-treated (ER^+, n = 90) and ER^- (n = 82) breast cancer (red, upregulated; blue, downregulated; §miRNAs also prognostic in triple-negative receptor [TNR], n = 37). **(C)** Primary (pri-)miRNA expression of the mature miRNA was correlated in other published cohorts for DRFS by meta-analysis. Summary hazard ratios and 95% CI for pri-miR-128a, pri-miR-210, pri-miR-548d and pri-miR-342 were 2.49 (1.44–4.32), 3.1 (1.43–6.74), 3.54 (2.06–6.09) and 0.44 (0.26–0.76), respectively (i.e., patients with high pri-miR-210 develop distant disease 2.5-times more frequently per unit time compared with patients with low expression; patients with high pri-miR-342 develop distant disease slower than those with low levels). Some of these pri-miRNAs were also prognostic for recurrence-free survival. **(D)** Expression levels of miRNA-processing genes were found to be prognostic for DRFS at Cox univariate analysis (e.g., patients with high Argonaute 2 had a hazard ratio of 2.63 for distal breast cancer relapse).
DRFS: Distant relapse-free survival; ER: Estrogen receptor; RFS: Relapse-free survival.

regard to pri-miRNA and target mRNA expression of the mature miRNAs identified. Eight published gene expression signatures were implemented as surrogate markers of biological processes in cancer (i.e., proliferation, estrogen receptor 1 and human EGF receptor 2 signaling, stem cell, invasion, immune response, apoptosis and hypoxia).

miRNA:mRNA target relationships

To identify functional miRNAs, several statistical methods were used to confirm strong anticorrelation of predicted mRNA targets (i.e., cumulative relative risk, predicted target signature score and regulatory effect score). These constraints were imposed to remove spurious matches and identify a subset

of tissue-specific, functional mRNA targets of a dysregulated miRNA [3], as there is a 'many-to-many relationship' between miRNAs and mRNAs, since a single miRNA targets multiple mRNAs and a single mRNA is regulated by multiple miRNAs [4].

Results

Identification of independently prognostic miRNAs in breast cancer

By including clinical, pathological and molecular factors, the two-step Cox analysis approach allowed the identification of miRNAs associated with clinical outcome or a specific covariate, independently from other miRNAs and covariates. miRNA signatures were able to effectively dichotomize patients into good and poor prognosis groups.

The miRNAs independently prognostic of DRFS in ER$^+$, ER$^-$ and triple-negative receptor subtypes are shown in Figure 1A & B. Two previously studied prognostic miRNAs were upregulated: miR-128 in ER$^+$ and miR-210 in ER$^-$ [5]. Three additional miRNAs were independently prognostic when all samples were included (high miR-29c and -642, good prognosis; high miR-548d, poor prognosis). Of note, other independent prognostic factors included: lymph-node disease, tumor grade; proliferation and hypoxia gene signatures in ER$^+$ patients; and hypoxia, invasion and immune response signatures in ER$^-$ patients. However, compared with these factors, miRNAs have the benefit of being very stable and can easily be measured in blood, biopsy or formalin-fixed paraffin-embedded samples.

Prognostic miRNA clusters in ER$^+$ & ER$^-$ breast cancer

miR-451 functions in a cluster with miR-144, and both were associated with good prognosis in ER$^+$ patients at univariate analysis, although high miR-144 also predicted poor prognosis in ER$^-$ cases. Thus the cluster has different roles in the two subtypes. The miR-24/27/23 cluster seems to have a specific role in only ER$^-$ patients and was associated with a poor prognosis in this group, but not in ER$^+$ patients (i.e., high miR-23b and -24 both present a hazard ratio >2 of distant relapse in all 207 BC patients; while miR-27b, 24 and 24-1* present a hazard ratio of 2.08, 3.63 and 5.23, respectively, in ER$^-$ patients).

Meta-analysis of coordinated pri-miRNA expression

The pri-miRNAs for six of the mature miRNAs identified (pri-miR-128a, -210, -29c, -342, -27b and -548d) were found to have coordinated expression in independent cohorts and many were concordantly prognostic (Figure 1C). This indicates transcriptional regulation of these miRNAs in BC, as opposed to modulation due to abnormal processing.

Prognostic miRNAs associated with pathways & biological processes in breast cancer

Significantly downregulated mRNA targets were identified as members of pathways dysregulated in BC, especially tumor growth and metastasis (Figure 1A). Major biological processes were associated with miRNA expression, including:

- High miR-210 with increased hypoxia, proliferation (independent of grade and ER status) and invasion. miR-210 was also associated with *ESR1* gene signature, even though raised miR-210 was prognostic in ER$^-$ patients;
- Low miR-150 with high immune response;
- High miR-27b with promotion of invasion;
- High miR-769-3p and high miR-144 with high proliferation;
- Low miR-135a/high miR-128a with ER positivity (the latter confirmed by both *ESR1* gene signature and immunohistochemistry).

However miRNA expression was still prognostic independent of these gene signatures.

The miR-210, -128 and -27b targets were then validated in independent BC datasets and found to be consistently downregulated and associated with worse prognosis (Figure 1A). In addition, downregulated mRNA targets (*ISCU*, *CBX7* and *IGF1R*) were correlated *in vitro* with high miR-210 expression and downregulation of NFE2L2 protein was measured by immunohistochemistry (target of miR-144).

miRNA-processing genes are dysregulated in breast cancer

As not all of the pri-miRNA levels matched with the mature expression, miRNA-processing genes were measured and found to also have prognostic significance (Figure 1D). Again, the mature prognostic miRNAs identified (Figure 1B) were still independently significant despite processing gene expression, reinforcing that the altered expression of these miRNAs is caused by transcriptional regulation as opposed to differential miRNA processing.

Interestingly, the disruption of miRNA production by depletion of Drosha, DCGR8 or Dicer has been shown to promote tumorigenesis [6–8] (e.g., Dicer is targeted by miR-103/107 leading to a reduction in mature miRNAs, including the miR-200 family, enhancing epithelial-to-mesenchymal transition and migration *in vitro*, as well as increased metastatic colonization *in vivo* [9]). In agreement with the current study, others have shown that upregulation of Argonaute 2 (AGO2), a component of the miRNA-induced silencing complex (miRISC) which is essential for miRNA-mediated repression, is associated with advanced tumors and distant metastases [10].

Expert commentary

miRNAs are consistently dysregulated in human cancers and have major roles as either oncogenes or tumor-suppressor genes. Bioinformatic analysis has greatly improved the ability to predict *bona fide* miRNA targets and is based on the evolutionarily conserved seed matches in the mRNA 3′ untranslated region. However, due to the sophistication of miRNA:mRNA interactions and the small effect exerted by an individual miRNA, few miRNA targets have been functionally validated [11]. The computational prediction programs developed can identify putative miRNA targets, but these suffer from high false-positive/-negative

readings due to the low-throughput nature of the query design (gene by gene or miRNA by miRNA) [12].

This large study has elegantly used microarray and next-generation sequencing technologies to determine the interactions between miRNA:mRNA as a whole network, rather than individual targets in BC. Crucially, clinicopathological factors, including survival outcomes, were also assessed. The data provided on specific miRNAs in ER^+ and ER^- breast cancer specimens could be the key to finding a specific target for ER-based therapies, and the prognostic nature of the miRNA signatures may outperform current validated tools [13,14].

Recently, several groups have investigated the combined analysis of miRNA–mRNA expressions in breast and other human cancers (Table 1). Enerly *et al.* have similarly identified biological processes in BC in which a 'pivot' miRNA plays a role, pointing to potential direct regulation by the miRNA [15]. Notably, miR-150 was again found to be upregulated and have strong enrichment of the immune response term among its positive correlated mRNAs and low levels were associated with poor prognosis, validating the current study [15]. Patients with colorectal cancer and low miR-150 also have poor survival and a worse response to adjuvant chemotherapy [16]. Interestingly, miR-150 has recently been shown to modulate the development of natural killer and invariant natural killer T-cell development in the innate immune system [17].

Although previously recognized, the role of hypoxia-inducible miR-210 (by HIFα) in BC is of great interest [18,19]. HIFα regulates genes involved in adaptation and protection against hypoxia; however, miR-210 regulates many genes that are not induced under hypoxia (e.g., oncogene *HOXA1*) [19]. Thus, HIFα is able to inhibit tumor growth through miR-210 regulation. Conversely, miR-210 expression is elevated in several cancers, and correlated with breast and melanoma metastasis [18].

Finally, in this analysis miR-27b was associated with the invasion gene signature and has been shown to stimulate cell migration/invasion in BC cells, although not solely by repression of tumor suppressor target *ST14* [20].

Five-year view

These data are a useful resource for future validation studies. From this study, the prognostic miR-128a, -27b and -210 showed evidence of cognate target downregulation, and expression of their targets was prognostic in a meta-analysis of several cohorts. Further insights into these miRNAs could be gained by performing advanced techniques such as high-throughout sequencing of RNAs isolated by crosslinking immunoprecipitation, which identifies functional protein–RNA interaction sites [21]. The miRNAs are coimmunoprecipitated with AGO proteins and bound RNA fragments sequenced by high-throughout methods, yielding genome-wide maps and functional insights, which could also be used to decode a precise map linking miRNA-binding sites to mRNA transcripts. This should provide a new approach to understanding the role of miRNAs in cancer biology. In addition, the maps allow target site determination for RNA interference therapy on clinically relevant mRNAs.

The main focus for the next few years will be delivering potential miRNAs to cancer patients, a task that has caused problems in the progress of siRNA treatments. The recent clinical success of a nanoparticle delivery system [22] and the continuing emergence of new technologies suggest that miRNA therapeutics is achievable.

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Key issues

- The study under evaluation is the largest combined miRNA:mRNA profiling in breast cancer, revealing some important functional roles.
- Ten prognostic miRNAs for distant relapse in breast cancer patients were identified in this study, including: miR-767-3p, -769-3p, -128a and -135a (all estrogen receptor-positive); and miR-27b, -144, -210, -342, -30c and -150 (all estrogen receptor-negative).
- Many of these miRNAs have been validated in a multicentric fashion (n >1000) by examining expression of their primary transcript and cognate mRNA targets.
- Clearly important candidates that should be considered for further investigation/therapeutic potential are miR-150, -210, -128a and -27b.
- Many important cancers, such as those of the lung, pancreas and prostate, are yet to be sequenced using this approach.

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EXPERT REVIEWS

Retinoblastoma protein determines aggressiveness in triple-negative breast cancer

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Jimmy Jacob*[1], Adam E Frampton[2], Leandro Castellano[1], Justin Stebbing[3] and Jonathan Krell[1]

[1]*Division of Cancer, Department of Surgery and Cancer, 5th Floor, MRC Cyclotron Building, Imperial College, Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK*
[2]*HPB Surgical Unit, Department of Surgery and Cancer, Imperial College, Hammersmith Hospital, Du Cane Road, London, W12 0HS, UK*
[3]*Department of Oncology, Imperial College Healthcare NHS Trust, Charing Cross Hospital, 1st Floor, E Wing, Fulham Palace Road, London, W6 8RF, UK*
**Author for correspondence: j.jacob@imperial.ac.uk*

Evaluation of: Arima Y, Hayashi H, Sasaki M *et al.* Induction of ZEB by inactivation of RB is a key determinant of the mesenchymal phenotype of breast cancer. *J. Biol. Chem.* 287(11), 7896–7906 (2012).

Retinoblastoma protein (RB) is one of the most important tumor suppressors and functions in multiple biological pathways that are deregulated during tumor initiation and progression. Epithelial-to-mesenchymal transition (EMT) is a reversible embryonic process by which epithelial cells lose cell–cell contact and polarity, and its aberrant activation can trigger tumor progression and metastasis. Previously, it has been shown that depletion of RB initiates EMT by downregulating the adhesion molecule E-cadherin. The evaluated article suggests that RB inactivation contributes to loss of cell cycle control and also leads to downregulation of the miR-200 family, thereby causing upregulation of ZEB expression and consequently EMT by downregulation of E-cadherin. RB inactivation could be a key event underlying the mesenchymal and aggressive phenotype of triple-negative breast cancer. Furthermore, exploring links between RB inactivation and EMT might reveal new therapeutic targets for triple-negative breast cancer.

Keywords: EMT • epithelial-to-mesenchymal transition • miR-200 • RB • retinoblastoma protein • TNBC • triple-negative breast cancer

Mutations of the retinoblastoma gene (*RB1*) resulting in loss of function have been identified in several human cancers, including those of the breast, lung and bladder, as well as in the rare inherited malignant tumor of the eye. Similarly, loss or reduction of *CDH1* (the gene encoding E-cadherin) is associated with increased invasion, metastasis and poor prognosis in several malignancies. Both retinoblastoma protein (RB) and E-cadherin are downregulated in the invasive and aggressive subtypes of breast carcinoma, but the relationship between these two proteins has remained uncertain.

Summary of methods & results

Retinoblastoma protein controls epithelial-to-mesenchymal transition in breast cancer cells

Depletion of RB tumor suppressor protein by siRNAs leads to downregulation of E-cadherin, disruption of cell–cell adhesion and induction of the mesenchymal marker vimentin in breast cancer cells, which are the hallmarks of the epithelial-to-mesenchymal transition (EMT) [1]. EMT contributes to the invasive and metastatic ability of cells, and infection of RB-negative breast cancer cells (e.g., BT549) with an adenovirus encoding human RB (Ad-RB) was found to cause mesenchymal-to-epithelial transition (MET) at the mRNA level, accompanied by a marked reduction in invasiveness. This reduction of invasiveness was also seen after infection of MDA-MB-231 (RB-inactivated) and MDA-MB-157 (poorly expressing RB) breast cancer cells with Ad-RB Am (an adenovirus encoding an active mutant of RB that is refractory to CDK-mediated phosphorylation).

RB is suppressed in mesenchymal breast cancer cells & patient samples

Mesenchymal breast cancer cells (e.g., MDA-MB-436, BT549, MDA-MB-157 and MDA-MB-231) were shown to express vimentin, fibronectin or N-cadherin and were devoid of E-cadherin (see Figure 1). In addition, cells with reduced RB expression were subsequently demonstrated to have high levels of p16 (an endogenous CDK inhibitor) through negative feedback. RB

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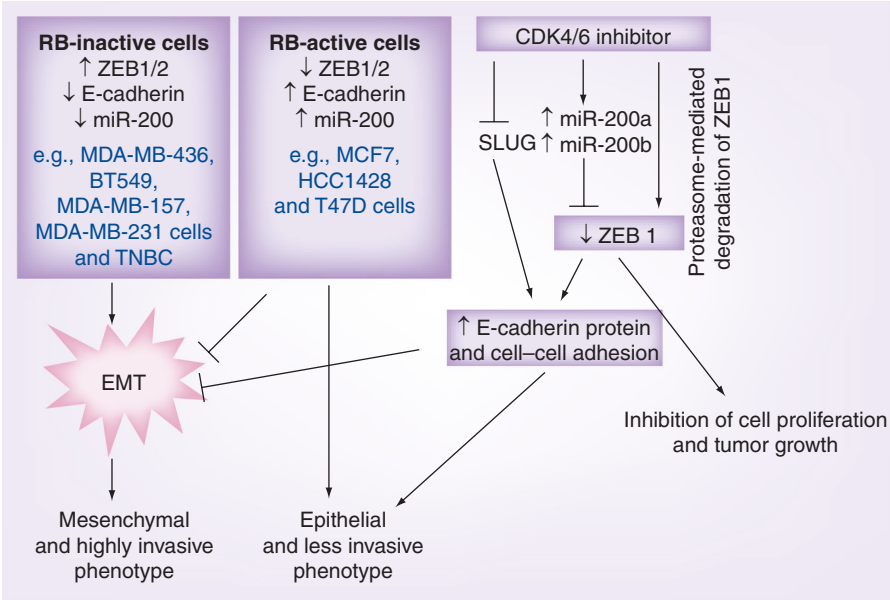

Figure 1. Retinoblastoma protein inactivation is associated with the mesenchymal phenotype in breast cancer. Cells and tissue samples (triple-negative breast cancer) with inactive RB have an EMT phenotype, which is more aggressive, invasive and metastatic. The CDK4/6 inhibitor PD0332991 is able to attenuate phosphorylation of RB and, via the induction of miR-200a/b, leads to downregulation of ZEB1 and increased transcription of the *CDH1* gene, which encodes the cell–cell adhesion molecule E-cadherin.
EMT: Epithelial-to-mesenchymal transition; RB: Retinoblastoma protein; TNBC: Triple-negative breast cancer.

CDK inhibition leads to a reduction in ZEB1

CDK inhibitors have potential as anticancer drugs. Using a Screening Committee of Anticancer Drugs inhibitor kit, a CDK4 inhibitor was demonstrated to induce cell cycle arrest and MET (by downregulation of ZEB1 and SLUG, another EMT-related transcriptional repressor) in MDA-MB-231 cells. The reduction of ZEB1 was found to be caused by increased levels of miR-200a and miR-200b, as well as by direct degradation by the proteasome.

The CDK4/6 inhibitor, PD0332991 (Pfizer), was shown to have even more profound effects on ZEB1 *in vivo*. Using an MDA-MB-231 orthotopic xenograft model in nude mice, oral administration of PD0332991 (n = 4) over a 2-week period resulted in a significant reduction in tumor growth compared with a vehicle control (n = 6). Given that this CDK inhibitor was able to reduce the expression of ZEB1 *in vitro* (as well as cell proliferation and invasiveness), this is the likely mechanism *in vivo*, providing further evidence for the potential use of such compounds in cancer therapy.

expression was next examined by immunohistochemical analysis in 18 TNBC patient specimens: RB was positive in 22% (four out of 18) of patients; inactivated in 28% (five out of 18) of patients and negative in 50% (nine out of 18) of patients.

RB/ZEB/miR-200 pathway controls EMT

RB depletion in epithelial breast cancer cells (e.g., MCF7, T47D and HCC1428) resulted in increased expression of ZEB1 and ZEB2 (transcriptional repressors of E-cadherin) and consequently EMT. This effect was completely inhibited by simultaneous knockdown of ZEB1 and ZEB2, indicating that these proteins are vital to RB-mediated EMT. In addition, as the miR-200 family members are known to target the ZEBs via a reciprocal feedback loop [2], forced expression of miR-200a/b was able to efficiently inhibit EMT in RB-depleted cells by targeting ZEBs. In RB-inactive/negative patient specimens, immunohistochemical analysis revealed that 43% (six out of 14) of patients expressed ZEB1, indicating that RB depletion may be required for ZEB1-induced EMT in triple-negative breast cancer (TNBC).

ZEB1 depletion induces MET

As seen in other tumor types, such as pancreas [3] and prostate [4], suppression of ZEB1 was able to reverse EMT (i.e., MET) with increased cell–cell adhesion and induction of epithelial markers in MDA-MB-231 breast cancer cells. This effect was stronger than for ZEB2 alone, but maximal with combined silencing of ZEB1 and ZEB2.

Discussion

TNBC refers to a subgroup of breast carcinomas that do not express estrogen receptors, progesterone receptors or HER2 and accounts for 15–20% of all breast cancer cases. Earlier studies showed that RB acts as a regulator of E-cadherin by binding to its promoter sequence in conjunction with the transcription factor activator protein-2A, but the inactivation of RB induces downregulation of the adhesion molecule E-cadherin, thereby inducing EMT and tumor progression [1]. Further studies revealed that RB inactivation leads to a sequence of molecular events culminating in EMT, including an increase in the expression of ZEB1 and ZEB2, which act as transcriptional repressors of E-cadherin. This study demonstrated that a similar effect was induced by downregulation of the miR-200 family, although the precise molecular association between RB and these miRNAs requires further evaluation [5]. A number of recent studies have described the role played by miRNAs in regulating EMT, and the miR-200 family has been shown to be vital to this process, in part owing to the ZEB/miR-200 double-negative feedback loop [6,7].

A recent study showed that expression of the miR-200a/141 cluster resulted in G1 arrest, due to increased p27/KIP1 and decreased CDK6 expression, but expression of the 200bc/429 cluster resulted in G2 arrest with a reduction of p27/KIP1 and upregulation of the inhibitory phosphorylation of CDC25C [5]. This suggests that downregulation of ZEB1 expression by CDK inhibitors in breast cancer cells demonstrated in the current study may be due to the reciprocal suppression of ZEB and the miR-200 family [5].

Adenovirus-mediated overexpression of RB in MDA-MB-231 and BT549 cells (both RB-inactive cells) resulted in a reduction of ZEB1 and ZEB2 mRNA, respectively, suggesting that RB transcriptionally suppresses the expression of ZEBs. Furthermore, it was observed that the reduction in ZEB1 expression induced by CDK inhibition was mediated by the ubiquitin–proteasome pathway. Interestingly, ZEB1 possesses an LXCXE RB-binding motif, which suggests that an E3 ubiquitin ligase may target the RB–ZEB1 complex.

In-depth studies involving the screening of CDK inhibitors that suppress ZEB1 expression revealed a CDK4/6 inhibitor (PD0332991) that decreased phosphorylation of RB on Ser807/811, induced cell cycle arrest and inhibited tumor growth. It was also shown that this effect was not due to cell cycle-dependent expression of ZEB1 or E2F1-mediated signaling. Furthermore, shRNA-mediated depletion of ZEB1 resulted in significant inhibition of cell proliferation, and treatment with PD0332991 suppressed cell invasiveness, which may be due to the inhibition of ZEB-mediated mesenchymal phenotypes. This study provides further evidence that ZEB1 expression is a potential marker of poor prognosis in breast cancer, as in several other tumors [8], and may serve as a predictive marker for the treatment of TNBC. However, further studies are necessary to determine whether the expression of ZEB1 influences the prognosis of TNBC patients.

Five-year view

TNBC is characterized by distinct molecular and histological features [9]. TNBC is generally associated with an unfavorable prognosis, but a subgroup of patients demonstrate an increased sensitivity to conventional chemotherapeutic agents [9]. Current attempts to improve prognosis for patients with TNBC entail the development and use of novel targeted agents in conjunction with optimizing the type and scheduling of currently available cytotoxic agents. An interesting clinical target in TNBC is the enzyme poly-ADP ribose polymerase (PARP), and treatment with the PARP inhibitor iniparib was associated with significant improvements in the rate of tumor regression, median progression-free survival and median overall survival in patients with TNBC when used in conjunction with gemcitabine and carboplatin [10]. Other potential targets in TNBC include the EGF receptor and VEGF [11]. However, new therapeutic targets are required to improve prognosis in these patients.

RB is one of the most studied proteins in cancer biology and has been shown to interact with over 100 other proteins, and its association with the E2F transcription factors has been particularly well described. The biological functions of RB include tumor suppression, regulation of the cell cycle, differentiation and apoptosis [12,13]. TNBC has distinct clinico–pathological features and falls within the basal-like histological subset of breast cancers, which frequently exhibit inactivation of RB [14]. EMT plays an important role in breast cancer metastasis, especially in TNBC. This particular study revealed that RB inactivation is one of the key events underlying the mesenchymal and aggressive phenotype of TNBC, and exploration of the molecular links between RB inactivation and EMT may thus reveal new targets for TNBC treatment. ZEB1 and ZEB2 are crucial EMT activators, whereas members of the miR-200 family induce epithelial differentiation. They are reciprocally linked in a feedback loop and strictly control the expression of one another. Proteins that could possibly regulate the miR-200 family in RB-inactive cells would be important in maintaining cellular plasticity and may represent future potential therapeutic targets [4]. This study shows that the CDK inhibitor PD0332991 suppressed ZEB1 expression and may be effective for the treatment of patients with TNBC. Recent clinical trials have proved the importance of PD0332991 in treating different cancers [15,16], which suggests that it could be a potential candidate for TNBC treatment and could provide the key to treating this lethal disease. Maintaining the status of RB in cancer treatment, or developing drugs that can mimic the function of RB, are important strategies that could be used to develop new cancer therapeutic targets with potentially fewer side effects [12]. Current and future studies will focus on the discovery of novel targets such as RB in TNBC and the design of agents to inhibit or promote their activity. Interestingly, in a recent deep sequencing of breast cancer, miR-210 was found to be upregulated in the transition from *in situ* to invasive ductal carcinoma, and several crucial breast cancer genes were found to be inversely related to its expression including *BRCA1*, *FANCD*, *FANCF*, *PARP1*, *CDH1* and *RB1* [17]. Furthermore, TNBC was characterized by activation of miR-200c, miR-128 and the polycistronic miR-17-92 cluster compared with other breast cancer subtypes.

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No writing assistance was utilized in the production of this manuscript.

Key issues

- Inactivation of retinoblastoma protein (RB) contributes to mesenchymal-like morphology with an invasive phenotype in breast cancer cells.
- RB is inactivated in clinical specimens of triple-negative breast cancer, suggesting that restoration of the function of RB could be a therapeutic solution.
- Identification of CDK inhibitors that induce downregulation of ZEB1 expression may show potential for triple-negative breast cancer treatment.
- The evaluated study provides links between RB, the miR-200 family and ZEB1/2; however, further investigation is required to understand the mechanisms involved.

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EXPERT REVIEWS

Determination of cut-offs for circulating tumor cell measurement in metastatic cancer

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Jimmy Jacob[1], Jonathan Krell[1], Leandro Castellano[1], Long R Jiao[2], Justin Stebbing[3] and Adam E Frampton[†2]

[1]*Dept. of Oncology, Dept. of Surgery and Cancer, 5th Floor, MRC Cyclotron Building, Imperial College, Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK*
[2]*HPB Surgical Unit, Dept. of Surgery and Cancer, Imperial College, Hammersmith Hospital, Du Cane Road, London, W12 0HS, UK*
[3]*Dept. of Oncology, Imperial College Healthcare NHS Trust, Charing Cross Hospital, 1st Floor, E Wing, Fulham Palace Road, London, W6 8RF, UK*
[†]*Author for correspondence: a.frampton@imperial.ac.uk*

Evaluation of: Pierga JY, Hajage D, Bachelot T *et al.* High independent prognostic and predictive value of circulating tumor cells compared with serum tumor markers in a large prospective trial in first-line chemotherapy for metastatic breast cancer patients. *Ann. Oncol.* DOI: 10.1093/annonc/mdr263 (2011) (Epub ahead of print).

The metastatic transformation of epithelial tumors progresses through various steps leading to the generation of circulating tumor cells (CTCs). Measurement of CTCs in the peripheral blood is being increasingly recognized as a promising tool in breast oncology. Several studies have evaluated the prognostic significance of CTCs in newly diagnosed metastatic breast cancer (MBC) patients. The IC 2006-04 was a high-powered, prospective, multicenter, observational study conceived to assess CTC changes in women with MBC treated with first-line chemotherapy. Levels ≥5 CTCs/7.5 ml blood at baseline and before the second cycle of treatment were independent prognostic factors associated with shorter progression-free and overall survival. This study provides further level II evidence for the clinical and prognostic value of CTCs in MBC, confirming data from earlier small studies. It also provides proof that CTCs should be investigated in ongoing interventional trials to see if better patient outcomes can be attained by altering treatment based on CTC levels.

KEYWORDS: CirCe01 • circulating tumor cells • IC 2006-04 • metastatic breast cancer • overall survival • progression-free survival • serum markers • SWOG-0500

Summary of methods & results

Methods

The primary objective was to investigate whether circulating tumor cell (CTC) counts between baseline and the second treatment cycle were associated with progression-free survival and overall survival. Secondary objectives were to analyze CTC counts between baseline and cycle 3/4 (C3/4), and to compare CTC counts to known serum tumor markers (carcinoembryonic antigen [CEA], carcinoma antigen [CA] 15-3 and lactate dehydrogenase). Additional data showing correlations between CTC counts and the serum marker CYFRA 21-1 were presented at the IMPAKT Breast Cancer Conference (May 2011) [1,2].

Inclusion criteria were: metastatic breast cancer (MBC) patients prior to first-line chemotherapy; subsequently treated with or without targeted therapy (e.g., depending on HER2 status); and with a life expectancy of at least 3 months. Prior hormone therapy for MBC was permitted. Blood tests and CTC counts were performed at baseline (C1), before each treatment cycle and at disease progression (data not shown). Radiological assessment of tumor response was carried out at baseline and before C3 or 4. A CTC prognostic threshold of ≥5 CTCs/7.5 ml was used [3–6].

The landmark study by Cristofanilli *et al.* identified that a ≥5 CTCs count before treatment was an independent predictor of progression-free survival (PFS) and in MBC [7]. The IC 2006-04 (2005) was specifically designed to confirm this. In the Cristofanilli study (n = 177), 175 patients were required to achieve 80% power (two-sided; $\alpha = 0.05$). Hence it was calculated for the IC 2006-04 that 216 patients

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Table 1. Recent and ongoing studies evaluating the prognostic value of circulating tumor cells in metastatic breast cancer.

Investigators/ institution/ study dates	Name/number of patients	Description	Measures	Main results	Ref.
Bidard FC, Pierga J-Y Institut Curie, Paris, France June 2007– September 2009	IC 2006-04 n = 267	Prospective and statistically powered cohort study CTC-related outcome assessed in a homogenous population of MBC patients treated with first-line chemotherapy	**Primary:** Determine whether changes in CTC count between baseline and second cycle of treatment is associated with progression-free survival at 6-months **Secondary:** Progression-free survival and according to CTCs at baseline and before cycle 3/4 of treatment. Comparison of CTCs with standard serum tumor markers (including CYFRA 21-1)	Baseline ≥1 CTCs in 65% and ≥5 CTCs in 44% Presence of ≥5 CTCs at baseline associated with bone ($p < 0.0001$) and liver metastases ($p < 0.03$) Baseline ≥1 or ≥5 CTCs are independent prognostic factors and double the hazard for disease progression at multivariate analysis ($p = 0.003$ and 0.006, respectively) Baseline ≥5 CTCs doubled the hazard for mortality during the study period ($p = 0.03$) compared with <5 CTCs. Prior to C2, ≥5 CTCs also associated with worse PFS and OS at 6 months (both $p = 0.0001$) CTCs had prognostic value independent of other serum markers CYFRA 21-1 was also associated with PFS ($p = 0.05$) at multivariate analysis	[1,2]
Pierga J-Y Institut Curie, Paris, France January 2010– January 2014	CirCe01 n = 568 (estimate)	Evaluation of the use of CTC to modify chemotherapy after the second-line of chemotherapy for MBC Phase III multicenter, randomized, open-label study Determination of CTC versus conventional clinical and radiological evaluation	**Primary:** Overall survival **Secondary:** Measure of safety and tolerability Improvement of quality of life Time to progression and time to discontinuation of therapy after third line Comparison of CTCs with standard serum tumor markers	Ongoing	NA
Smerage JB, Hayes DF, Winer EP Southwest Oncology Group, USA October 2006– September 2011	SWOG-0500 n = 500 (estimate)	Randomized Phase III trial to test the strategy of changing chemotherapy versus maintaining chemotherapy for MBC CTCs evaluated at baseline and after one cycle of first-line chemotherapy Those with persistently elevated CTC count (≥5 CTCs/7.5 ml) will be randomized to either continue initial therapy until clinical/ radiographic evidence of disease progression or switch to a different chemotherapy	**Primary:** Overall survival Progression-free survival Prognostic value of sequentially collected CTC values **Secondary:** Comparison of CTCs with standard serum tumor markers	Ongoing	[101]

CTC: Circulating tumor cell; EMT: Epithelial-to-mesenchymal transition; G-CSF: Granulocyte colony-stimulating factor; HDCT: High-dose chemotherapy; MBC: Metastatic breast cancer; NA: Not available; SWOG: Southwest Oncology Group.

Table 1. Recent and ongoing studies evaluating the prognostic value of circulating tumor cells in metastatic breast cancer (cont.).

Investigators/ institution/ study dates	Name/number of patients	Description	Measures	Main results	Ref.
Gao H, Ueno NT University of Texas MD Anderson, TX, USA 2007–2010	Prognostic value of CTC in patients undergoing autologous stem cell transplantation for metastatic breast cancer n = 21	Prospective, Phase II study to test whether the presence of CTCs predicted worse survival in patients undergoing autologous stem cell transplantation for metastatic breast cancer; G-CSF given to mobilize hematopoietic stem cells and collection by apharesis; Isolation of $CD34^+$ hematopoietic stem cells and transfused back to the patient after HDCT; CTC count before G-CSF and 1-month post-transplantation; during mobilization of tumor cells and also in cells undergoing EMT	**Primary:** Determine whether G-CSF can mobilize tumor cells Determine whether the presence of CTCs before and after autologous stem cell transplantation can predict the clinical outcome	Out of the 21 patients, six had ≥1 CTC/7.5 ml blood prior to transplantation and nine had ≥1 CTC 1 month after the procedure G-CSF appears to recruit CTCs from bone marrow into the blood MBC patients with CTC count (≥5 CTCs) before or after HDCT followed by autologous stem cell transplantation had poor PFS and apharesis product can contain tumor cells and a subset have EMT features Patients with high EMT-related transcription factors and higher number of $CD326^+$ epithelial (tumor) cells in the residual apharesis product had shorter PFS Novel therapies are needed to target EMT in order to achieve better clinical outcomes in MBC	[19,102]
Giuliano M, Cristofanilli M University of Texas MD Anderson, TX, USA September 2004–November 2009	CTCs as prognostic and predictive markers in metastatic breast cancer patients receiving first-line systemic treatment n = 235	Retrospective evaluation of CTC count in newly diagnosed MBC patients before receiving first-line chemotherapy (45%; n = 235 of 517 consecutive MBC patients); Only 144 patients (61%) were available for follow-up CTC count; Lack of randomization and limited number of patients	**Primary:** Baseline CTC count correlated with PFS and OS (using cut-offs <5 CTCs/7.5 ml as favorable versus ≥5 CTCs/7.5 ml as unfavorable) Predictive value of baseline CTCs with different types of systemic therapy	Baseline <5 CTCs 60% versus ≥5 CTCs in 40% ≥5 CTCs at baseline associated with ≥3 metastatic sites (45.7 vs 29.8%; p = 0.011) Patients with CTCs <5 and ≥5; median PFS 12.0 and 7.0 months; median 40.1 and 21.9 months, respectively (both p < 0.001) Multivariate analysis: baseline CTCs an independent predictor of PFS and, regardless of hormone receptor status, HER2 status, location or number of metastatic sites Reduction to <5 CTCs from high baseline: chemotherapy plus bevacizumab (94%), chemotherapy plus HER2-targeting drugs (100%), endocrine treatment (10%), chemotherapy alone (50%) (although patient numbers in each group were small) In patients with HER2 overexpressed tumors receiving trastuzumab or lapatinib, baseline CTCs ≥5 was no longer prognostic as these patients appeared to have PFS benefit with this treatment In patients on taxane-based chemotherapy plus bevacizumab, baseline CTCs <5 not statistically associated with longer PFS or suggesting a therapeutic benefit for patients with ≥5 CTCs receiving this combination	[6]

CTC: Circulating tumor cell; EMT: Epithelial-to-mesenchymal transition; G-CSF: Granulocyte colony-stimulating factor; HDCT: High-dose chemotherapy; MBC: Metastatic breast cancer; NA: Not available; SWOG: Southwest Oncology Group.

with ≥5 CTCs at baseline were needed to see a 35% difference in 6-month PFS rates between those women with <5 CTC and ≥5 CTCs before C2. The accrual was extended to 267 patients as there were fewer patients with ≥5 CTCs at baseline, consistent with the Cristofanilli study.

Results

Results at baseline

Circulating tumor cells effectively predicted worse outcomes in MBC (Table 1). A baseline, CTC count ≥5 was an independent prognostic factor for PFS and in multivariate analysis with a relative risk of disease progression of 1.9 (95% CI: 1.2–2.8; p = 0.003) and mortality of 2.4 (95% CI: 1.1–5.4; p = 0.03). The presence of a single CTC at baseline was an independent predictor of PFS but not (relative risk [RR]: 1.9; 95% CI: 1.1–3.0; p = 0.006). PFS was double in women with no CTC at baseline (19.9 months) compared with those with CTC positivity.

Results prior to second cycle of treatment

A CTC count of ≥5 after C1 was associated with reduced 6-month PFS rates (C1 >5/C2 >5 64% vs C1 >5/C2<5 75%; p = 0.0001). Median PFS was 6.9 and 12 months, respectively and was significantly different between the two groups (C1 >5/C2 >5 95% vs C1 >5/C2 <5 78%; p = 0.0001). At 2 years, all patients with C2 >5 had disease progression compared with 71% of those with <5 CTC. A total of 89% with <5 CTC at baseline and C2 were still alive at 2 years compared with 60 and 31% with C1 >5/C2 <5 and C1 >5/C2 >5, respectively.

Serum markers including CYFRA 21-1

Circulating tumor cells had prognostic value independent of other serum markers. A total of 26% of patients had false-negative results with both CEA and CA 15-3 at baseline. A raised baseline CEA predicted PFS (RR: 2.0: 1.2–3.1; p = 0.002). For patients with one raised serum marker and ≥5 CTCs at baseline, there was no significant difference in the 6-month PFS predictive value of any of these biomarkers.

Breast carcinoma expresses cytokeratin (CK) 19 fragments in primary and metastatic lesions. CYFRA 21-1 is a marker that consists of two monoclonal antibodies reacting specifically against fragments of CK 19 in the serum. CYFRA 21-1 has been shown to have high sensitivity for recurrent primary disease and MBC with strong prognostic value [8]. CTC and CYFRA 21-1 were correlated with other serum markers, tumor burden, performance status and number of metastatic sites, but were independent of tumor subtype. CYFRA 21-1 was also associated with PFS (p = 0.05) at multivariate analysis [2].

Response to treatments

Circulating tumor cells must be used cautiously to monitor treatment response. First-line treatment was less heterogeneous than previously reported [7]. Notably, 47% of patients received bevacizumab plus chemotherapy in this study. After one treatment cycle, 90% of patients had stable or lower CTC counts. Chemotherapy plus anti-HER2 therapy or bevacizumab was associated with a statistically nonsignificant (p = 0.14) increase in the rate of reduction in CTC counts below 5, compared with chemotherapy alone (83, 64 and 53%, respectively).

Circulating tumor cell count could not predict radiological complete/partial response (CR/PR) to treatment at baseline or C2, but the ≥5 CTCs threshold could differentiate between CR/PR and stable or progressive disease at the time of radiological evaluation (i.e., C3/4; p = 0.02). Patients with ≥1 CTC at C2 were predicted to have progressive disease at C3/4. This was not the case at baseline or C3/C4. CTCs were not shown to be predictors of tumor response to therapy, but may predict progression earlier than radiological evaluation.

Discussion

Circulating tumor cells are a rare peripheral cell population; however, their isolation and enumeration is achievable using the CellSearch™ system (Veridex, NJ, USA). This allows automated enrichment and immunocytochemical detection for CTCs. Several studies have found CTCs both in patients with declared metastatic disease, and also in patients with nonmeasurable MBC [9]. Early detection of CTCs can serve as a predictor of metastases [3–5,7,10] and provide significant prognostic information about patients with MBC, but it is still up to the clinician to interpret this information. The relevance of a single CTC at follow-up remains uncertain. Initial results from the Simultaneous Study of Docetaxel-Gemcitabine Combination adjuvant treatment, as well as Extended Bisphosphonate and Surveillance (SUCCESS) trial (n = 2026) demonstrated that the presence of a single CTC in early breast cancer predicted poor disease-free, distant disease-free, and overall survival at more than 3 years follow-up, confirming the independent prognostic relevance of CTC counts [11]. The ideal CTC data should contribute to a decision in real practice that results in a more favourable clinical outcome for the patient, including increased overall survival, improved disease-free/progression-free survival, enhanced quality of life or a medico-economic benefit [12]. Ongoing randomized interventional trials have been designed to demonstrate the usefulness of CTC count in MBC (Table 1). CTC levels might enable personalization of therapies at all stages of breast cancer by better identifying high-risk patients and allowing clinicians to implement additional treatments when necessary.

Five-year view

Circulating tumor cells are rapidly becoming one of the key biomarkers for understanding metastasis and have the potential to provide better insights into tumor biology by allowing a 'blood biopsy' for various epithelial cancers. Characterization of CTCs in metastatic cancer patients could provide additional information to enhance their management. Whilst the CellSearch system is the most widely used detection method, newer technologies are becoming available. A high-throughput microfluidic mixing device, the herringbone-chip (HB-chip), has been demonstrated to provide an enhanced platform for CTC isolation. The HB-chip uses calibrated microfluidic flow patterns to drive cells into contact with the antibody-coated walls of the device;

this CTC-chip provides improved yield and purity of captured CTCs, preserving viability and allowing for detailed molecular and functional characterization [13]. Eventual gene expression profiling of CTCs may allow new potential therapeutic targets to be realized [14]. One study has isolated >100 CTCs from three metastatic patients (colorectal, prostate and breast) and, using the RNA extracted from CTC-enriched and CTC-depleted portions, have performed global gene-expression profiles, generating a list of cancer-specific, CTC-specific genes [15]. Recently, Flores *et al.* demonstrated the feasibility of molecular profiling of CTCs from patients with MBC using FISH [16]. That study assessed the *HER2* gene amplification status of CTCs and compared it with the primary and metastatic tissue. Interestingly, in patients with HER2-negative primary cancers, 33% had CTCs with clear amplification of the *HER2* locus. Finally, while the transcriptomic analysis of nonmetastatic breast cancers according to CTC detection has been unsuccessful [17], the processing of low numbers of CTCs in a high background of leukocytes is now possible and has allowed mRNA and miRNA profiling in MBC patients [18].

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Key issues

- First study to be prospectively designed and statistically powered for reporting circulating tumor cell (CTC)-related outcome as the primary measure in metastatic breast cancer (MBC) patients treated with first-line chemotherapy.
- CTCs are clinically valuable and an independent prognostic marker in newly diagnosed MBC (level II evidence).
- Persistently high CTC count (≥5 CTCs/7.5 ml) after initial treatment is an early predictive marker of poor PFS and OS (doubles hazard for both), even before radiological evaluation.
- There is some evidence that ≥1 CTCs/7.5 ml threshold can be used to define patients at high risk: patients with ≥1 CTC at baseline have shorter PFS and patients with ≥1 CTC after initial treatment are predicted to have progression at time of radiological evaluation; this differs from metastatic colorectal cancer where <3 CTCs/7.5 ml has been correlated with an unfavorable prognosis.
- Presence of CTCs seem to predict prognosis at any time during the treatment of MBC, raising the possibility that this measurement will allow proper staging of the disease.
- CTC results had prognostic value independent of other serum tumor markers (carcinoembryonic antigen, carcinoma antigen 15-3 and LDH); CYFRA 21-1 associated with PFS.
- Unable to demonstrate that CTCs can evaluate tumor response to therapy in MBC (awaiting results of ongoing interventional trials).
- Efforts are being made to perform molecular and functional characterization of CTCs; this might contribute to a greater understanding of cancer metastasis, the role of cancer stem cells and epithelial-to-mesenchymal transition
- Provides evidence that CTCs can be further investigated to develop personalized treatment regimens aiming for improved outcomes in MBC patients.

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