Hypoxic regulation of microRNA biogenesis

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4EBP1	eIF4E-binding proteins
AGO2	Argonaute 2
ALDOA	Aldolase A
AMPK	AMP-activated protein kinase
ANGPTL4	Angiopoietin-like 4
AP-1	Activating protein 1
APS	Ammonium persulphate
ARNT	Hydrocarbon receptor nuclear translocator
ARS2	Arsenite-resistance protein 2
Asn-803	Asparagine residue 803
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BCL2L11	Bcl-2 Like 11
CAIX	Carbonic anhydrase IX
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CBC	Cap-binding complex
CBP/p300	Cyclic adenosine monophosphate (cAMP)-response-element-binding protein (CREB) binding protein
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
ChIP-Seq	ChIP coupled with next generation high throughput sequencing
CLL	Chronic lymphocytic leukaemia
COX-2	Cyclooxygenase-2

C-P4H1	Collagen prolyl-4-hydroxylase
CREB	Cyclic AMP response element binding protein
CXCR4	C-X-C chemokine receptor type 4
DDX17 (p72)	DEAD box RNA helicase
DDX5	DEAD/H box 5
DDX5 (p68)	DEAD box RNA helicase
DFO	Desferrioxamine
DGCR8	DiGeorge syndrome critical region 8
DMSO	Dimethly sulfoxide
DNA	Deoxyribinucleic acid
dsRNA	Double stranded RNA
DTT	Dithiothreitol
DUF283	Unknown Function 283
dUTP	Deoxyuridine triphosphate
ECL	Enhanced chemiluminescence
EDN1	Endothelin 1
EDTA	Ethylenediaminetetraacetic acid
eEF2	Eukaryotic elongation factor 2
eEF2K	eEF2 kinase
EGR-1	Early growth response-1
EGRF	Epidermal growth factor receptors
eIF2α	Eukaryotic initiation factor 2α
EMT	Epithelial to mesenchymal transition
EPAS1	Endothelial PAS domain protein
EPO	Erythropoietin
ERα	Estradiol

ETC	Electron transfer chain
$FADH_2$	Flavin adenine dinucleotide
FBS	Fetal bovin serum
FIH-1	Factor inhibiting HIF-1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GFP	Green floursence protein
GLUT	Glucose transporters
GTP	Guanosine triphosphate
GW182	Glycine tryptophan repeat containing protein of 182 kDa
HIF:	Hypoxia inducible factor
hnRNP A1	Heterogenous nuclear ribonuclear protein
HNSCC	Head and neck squamous cell carcinomas
H-Ras	Transforming protein p21
HREs	Hypoxia responsive elements
HRMs	Hypoxically regulated miRNAs
Hsp70 and Hsp 90	Heat shock proteins
HuR protein	Human antigen R protein
HUVEC	Human umbilical vein endothelial cells
IGF2	Insulin-like growth factor ii
IL-8	Interleukin 8
IPAS	Inhibitory PAS domain
ISCU1/2	Iron-sulphur cluster assembly protein
K-Ras	V-Ki-ras2 Kirsten rat sarcoma viral oncogene
L1CAM	L1 cell adhesion molecule
LOX	Lysyl oxidase

MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblasts
miRNA	microRNA
miRtrons	Short intronic hairpins
MNT	Max binding protein
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MYC (c-MYC)	Myelocytomatosis oncogene
NADH	Nicotinamide adenine dinucleotide
NC	Negative control
NF-κB	Nuclear factor kappa light chain enhancer activated B cells
ODD	Oxygen dependent degradation domain
$p70^{s6k}$	Protein Ser-Thr kinase that phosphorylates the ribosomal S6 subunit,
PACT	Protein activator of PKR
PAS domain	Per/Arnt/Sim domain
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor B
PDK1	Pyruvate dehydrogenase kinase 1
PERK	Endoplasmic reticulum resident kinase
PHD1	Prolyl hydroxylase domain 1
PHD2	Prolyl hydroxylase domain 2
PHD3	Prolyl hydroxylase domain 1
PI3K	Phosphoinositide 3-kinase
PIWI	P-element induced wimpy testis

PMSF	Phenylmethylsulfonyl fluoride
POLR3K	Polymerase (RNA) III (DNA directed) polypeptide K
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
Pro-402 and Pro-564	Proline residue-402 and 564
PVDF	Polyvinylidene difluoride
pVHL	Von Hippel Lindau protein
PWM	Position weight matrix
RAD52	RADiosensitive protein 52
Ran-GTP	Ran guanosine triphosphate
RCC	Clear cell renal carcinoma
RISC	RNA induced silencing complex
RL	Renilla luciferase
RLC	RISC loading complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNU6B	U6B small nuclear RNA
RPC5	RNA polymerase III subunit C5
rpS6,	Ribosomal protein S6
RT PCR	Reverse transcription polymerase chain reaction
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNAs	Short hairpin RNA
SIP1	SMN interacting protein 1
siRNA	Small interfering RNA
ssRNA	Single stranded RNA

TAF9B	Transcription initiation factor TFIID subunit 9B
TARBP2	Tar binding protein 2
TEMED	Tetramethylethylenediamine
TP53	Tumour protein 53
tRNAs	Transfer RNA
UTR	Untranslasted region
VA1 RNA	Adenoviral RNA
VEGF	Vascular endothelial growth factor
XPO5	Exportin 5
Y-RNAs	Non-coding cytoplasmic localized RNAs
ZEB1	Zinc finger E box-binding homeobox 1

Abstract

Hypoxia is a key feature of many cancers and the presence of hypoxia is associated with more aggressive and metastatic tumours. MicroRNAs are 17-22 nucleotides, non-coding, single stranded RNA that are important regulators of gene expression. Functional studies show that microRNAs are involved in regulating many cellular processes including developmental timing, cell differentiation, cell proliferation and cell death. The expression levels of many microRNAs are deregulated in human disease conditions including cancer. In addition to deregulation of specific microRNAs in cancer, it has emerged that most tumour cell lines and cancers are characterised by global reductions in microRNA expression when compared to adjacent normal tissue. Cancers are commonly characterised by hypoxia and also by global reductions in the levels of mature microRNAs.

This thesis examined the hypothesis that hypoxia mediates the global reduction of microRNAs through repressive effects on microRNA biogenesis proteins. Cancer cell lines were exposed to hypoxia and manipulations of hypoxia inducible factor (HIF) and HIF hydroxylase activity. The effects of hypoxia on the mRNA and protein levels of enzymes involved in microRNA biogenesis (DICER, DROSHA, TARPB2, DCGR8, XPO5) were determined by RT PCR and immunoblotting. The effect of hypoxia on microRNA biogenesis and function was determined with microarrays, RT PCR, activity assays and reporter assays.

In two breast cancer lines (MCF7 and SKBR3), a colorectal cancer cell line (HT29) and a non-cancer cell line (HUVEC) there were significant reductions of DICER mRNA and protein levels after exposure to hypoxia. This effect was independent of HIF but dependent on the HIF hydroxylase PHD2 and was partly mediated by feedback effects by microRNAs. Furthermore, several other proteins with critical

roles in microRNA biogenesis such as DROSHA, DGCR8, TARBP2 and XPO5 also showed significant and co-ordinated repression under hypoxic conditions. The significant and consistent reduction in the levels of proteins with central roles in microRNA biogenesis under hypoxia did not have a substantial effect on the expression levels of mature microRNAs over the time course of these experiments. Even though hypoxia exerted only modest effects on the production of mature microRNAs, a significant influence of hypoxia on the function of exogenously introduced precursor microRNA was observed. These observations provide further and important interfaces between oxygen availability and gene expression and a potential mechanistic explanation for the reduced levels of microRNAs observed in some cancers. They provide further support for the existence of feedback mechanisms in the regulation of the microRNA biogenesis pathway and the relative stability of microRNAs.

Declaration

'I certify that this work does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text';

Kanchana V. Bandara

Publications and presentations

Peer reviewed publications-submitted

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Chapter 1 Introduction

1.1 Hypoxia

Hypoxia is a reduction in the normal level of tissue oxygen (O_2) tension, and it is a key feature of chronic vascular disease, pulmonary disease and cancer. Tissue hypoxia can have widespread effects on cell functions, as O_2 availability is critical for many cellular processes. O_2 plays a major role in several important enzymatic reactions such as the activity of oxidases, hydroxylases and oxygenases. Most importantly, O_2 is essential for the mitochondrial oxidative phosphorylation and adenosine triphosphate (ATP) production.

In aerobic conditions glucose metabolism involves production of electron carriers, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) from NAD+ and FAD respectively, through the citric acid cycle following the initial steps of glycolysis (conversion of glucose to pyruvate) and oxidative decarboxylation (conversion of pyruvate to acetyl coenzyme A). The transfer of electrons from NADH or $FADH_2$ to O_2 through the electron transfer chain (ETC) regenerates NAD+ and FAD, and energy released during the ETC is converted to ATP in the mitochondria (Rich, 2003). In hypoxia, the lack of a recipient (i.e. O_2) for the final transfer of electrons prevents the regeneration of NAD+ and FAD. Due to the reduction of these electron acceptors the citric acid cycle is interrupted, and oxidative decarboxylation of pyruvate does not take place. Therefore, pyruvate is metabolised through an anaerobic glycolytic pathway in the cytoplasm and is converted into lactic acid, producing a much lower yield of ATP per glucose molecule (Rich, 2003). Thus, without an adaptive response to hypoxia most cell functions are compromised. Therefore, mechanisms by which cells sense and respond to low O_2 are essential for cell survival. These mechanisms involve

coordinated regulation and expression of many genes critical for the adaptation to hypoxia.

1.2 Hypoxia and cancer

Hypoxia has emerged as a central feature in many tumours. During tumour development, some areas of solid tumours can become hypoxic due to the distortion caused by rapid cellular proliferation and aberrant blood vessels. Thus, resulting reduced O_2 diffusion to parts of the tumour, with increasing distance from the vasculature (Reynolds et al., 1996, Thomlinson and Gray, 1955, Vaupel et al., 1989). Normally mammalian tissue O_2 levels can vary from 2% – 9% (on average 40 mmHg); however, solid tumours contain regions with O_2 levels as low as 0.7% (approximately 5 mmHg) (Brown and Wilson, 2004, Bertout et al., 2008), or regions completely devoid of O_2 (referred to as anoxic tissue) (Bertout et al., 2008). Prolonged, limited oxygen diffusion in tumours result in chronic hypoxia, while acute hypoxia may be caused by a transient block in a blood vessel (Chaplin et al., 1986).

Early evidence of hypoxic regions in tumours was reported by Thomlinson and Gray in the 1950s. Looking at histological sections of human lung cancers they reported that tumours grew as cords around blood vessels and the larger cords contained a necrotic core (Thomlinson and Gray, 1955). They observed that cells 180 μm away from a blood vessel were necrotic and this was close to the approximate calculated distance of O_2 diffusion (145 μm) before being metabolised by the tissue (Thomlinson and Gray, 1955). Bordering the necrotic core were layers of viable cells, and they observed an O_2 gradient with normal O_2 levels closer to a blood

vessel, decreasing gradually to hypoxic or anoxic levels towards the necrotic centre (Thomlinson and Gray, 1955).

Initially, levels of hypoxia in tumours were measured using oxygen electrodes and since then many more methods such as *in vivo* polarographic measurements (Montgomery et al., 1950, Montgomery and Horwitz, 1950) and *ex vivo* cryospectrophotometric measurements have been developed with the hope of finding a non invasive, but a precise method (Kallinowski et al., 1990, Vaupel et al., 1991, Brizel et al., 1994). Imaging of increased glycolysis, or other compounds that accumulate in hypoxic tumours using positron emission tomography has allowed the monitoring of hypoxia by a less invasive but indirect method (Bertout et al., 2008, Rajendran et al., 2004). Using oxygen electrodes early researchers showed that the average O_2 tension in tumour tissues was much lower than in normal tissues (Vaupel et al., 1989, Kolstad, 1968). The average O_2 pressure in breast tumours (10 mmHg) were found to be a lot lower than normal breast tissue (60 mmHg) (Vaupel et al., 2004). In cervical cancers tissue hypoxia was shown to increase with the severity of the disease stage, with tissue hypoxia increasing as the disease progressed from early stages to stage 3 cancers (Kolstad, 1968). Tumour hypoxia is associated with increased metastasis and poor prognosis in patients suffering from squamous tumours of the head and neck, as well as cervical and breast cancers (Fyles et al., 2002, Harris, 2002, Eschmann et al., 2005, Nordsmark et al., 2005).

Hypoxia in solid tumours makes radiation therapy (Gray et al., 1953) and other anti-cancer therapies ineffective by increasing resistance in the cancer cells (Brown, 1999, Thomlinson and Gray, 1955). In radiotherapy, cells are exposed to ionising radiation, which can generate free radicals in DNA or water molecules in cells. These

free radicals will rapidly react with O_2 and generate a peroxy radical resulting in a chemically modified DNA molecule (in the form of DNA double strand breaks, DNA single strand breaks, DNA base modifications and DNA-DNA and DNA-protein cross-links) (Bristow and Hill, 2008). Due to reduced O_2 levels in hypoxic cells most of the free radicals in DNA will not form peroxy radicals and can be restored to the undamaged form by hydrogen donation from non-protein sulfhydryls in the cells (Gray et al., 1953, Brown, 1999). Hypoxic cells are roughly three times more resistant to radiation induced damage compared to well oxygenated cells (Frankenberg-Schwager et al., 1991, Terris et al., 2002, Bertout et al., 2008, Bristow and Hill, 2008).

Usually cells can not survive under hypoxic conditions for prolonged periods: however cancer cells can undergo genetic changes that will allow them to adapt to hypoxia (Harris, 2002). The genetic changes that occur as adaptations to hypoxia enable the cells to overcome nutritive deprivation, and selection of these genetically altered cells further increases the malignant progression and more aggressive treatment resistant disease (Hockel and Vaupel, 2001a, Hockel and Vaupel, 2001b, Duffy et al., 2003, Vaupel and Harrison, 2004).

1.3 Cell response to hypoxia

Cells respond to changes in O_2 tension with multiple molecular and cellular mechanisms, including alteration in metabolic pathways, as well as changes in gene regulation through transcriptional and translation mechanisms. In hypoxia the cellular metabolism change from aerobic to anaerobic processes (known as the Pasteur effect) (Racker, 1974, Dang and Semenza, 1999). In very early work, Otto

Warburg demonstrated that cancer cells favoured glycolysis even in well oxygenated conditions (Warburg effect) (Warburg, 1956). Changes in O_2 tension in tissues can result in the induction of specific genes involved in O_2 homeostasis, such as erythropoietin (*EPO*) (to increase haemoglobin production) or vascular endothelial growth factor (*VEGF*) (to increase blood vessel formation) in mammalian cells.

Gene regulation in hypoxia is mediated by transcriptional and post-transcriptional mechanisms. Transcriptional gene regulation in hypoxia is mainly mediated by the transcription factor hypoxia inducible factor-1 (HIF-1) (detailed discussion on HIF-1 can be found below) (Wang and Semenza, 1995, Wang and Semenza, 1993, Wang et al., 1995). Post-transcriptional gene regulation in hypoxia is mediated through influencing mRNA stability and protein translation (Liu et al., 2006, Goldberg et al., 1991). Some hypoxically induced genes such as *EPO* are regulated at the transcriptional as well as post-transcriptional levels, where the mRNA levels are maintained through increased transcription and increased mRNA stability (Goldberg et al., 1991). Another example of mRNA stability in hypoxia was observed in the hypoxically induced *VEGF* mRNA, where the mRNA half-life was increased in hypoxia (Levy et al., 1996). Binding of a hypoxically induced RNA binding protein HuR (Human antigen R) to the 3'UTR of the *VEGF* mRNA increased the stability of mRNAs in hypoxia when compared to normoxia (Levy, 1998, Levy et al., 1996, Levy et al., 1998).

Protein synthesis is reduced in cells exposed to hypoxia as a means of energy conservation (Romero-Ruiz et al., 2012, Pettersen et al., 1986, Hochachka et al., 1996). Critical steps in regulating protein synthesis in hypoxia involve: the phosphorylation of translation initiation factor eIF2α (eukaryotic initiation factor 2α);

the inhibition of the elongation factor eEF2 (eukaryotic elongation factor 2) and suppression of mTOR (mammalian target of rapamycin), and consequently its targets eIF4E-binding proteins (4EBP1), protein Ser-Thr kinase that phosphorylates the ribosomal S6 subunit ($p70^{s6k}$) and Ribosomal protein S6 (rpS6), which result in decreased mRNA translation, thus decreased protein synthesis (Koumenis et al., 2002, Liu et al., 2006, Romero-Ruiz et al., 2012, Wouters and Koritzinsky, 2008). The phosphorylation of the eIF2α at Ser51 is reversible upon re-oxygenation and is mediated by the hyperphosphorylated PERK (endoplasmic reticulum resident kinase) in hypoxia (Koumenis et al., 2002). Inhibition of the elongation factor eEF2 in acute hypoxia occurs due to the phosphorylation at Thr56, this process is also reversible upon re-oxygenation and is mediated by the prolyl hydroxylase PHD2 (Romero-Ruiz et al., 2012). In addition, in chronic hypoxia eEF2 is inhibited by phosphorylation at Ser-398 by eEF2K (eEF2 kinase) which is activated by AMPK (AMP-activated protein kinase) (Ryazanov et al., 1988) (Browne et al., 2004).

1.3.1 Hypoxia inducible factor-1 (HIF-1)

The hypoxia inducible factor (HIF-1) is the main transcription factor involved in regulating responses to changes in tissue oxygenation (Wang and Semenza, 1995). HIF was first identified as the main regulator of erythropoietin expression in hypoxia (Wang et al., 1995, Wang and Semenza, 1995). HIF-1 is a heterodimer consisting of two subunits; an α subunit (HIF-1α, HIF-2α and HIF-3α) and a β subunit (HIF-1β) also known as the hydrocarbon receptor nuclear translocator (ARNT) (Wang and Semenza, 1995, Wang et al., 1995). Oxygen tension in the tissue affects the expression and stabilisation of HIF-α subunits, whereas the HIF-β subunit is constitutively expressed (Wang and Semenza, 1993). These subunits are basic helix-loop-helix proteins that contain a PAS (Per/Arnt/Sim) domain.

The HIF-1α subunit is expressed in all tissues while HIF-2α and HIF-3α expression is limited to some tissues. HIF-2α (also known as endothelial PAS domain protein (EPAS1)) is also involved in gene regulation by binding to hypoxia response elements (HREs), and mainly expressed in vascular endothelium, liver parenchyma, lung type II pneumocytes and kidney epithelial cells (Wiesener et al., 2003[Tian, 1997 #6947, Harris, 2002, Tian et al., 1997). HIF-3α (also known as inhibitory PAS domain (IPAS)) is a dominant negative regulator of HIF1α and HIF-2α regulated transcription (Makino et al., 2001). HIF-3α is mainly expressed in the thymus, cerebellar Purkinje cells and the corneal epithelium of the eye (Makino et al., 2001, Gu et al., 1998).

At low oxygen levels, HIF-α is stabilised and accumulates while it is rapidly degraded in normoxic conditions (Figure 1.1) (Wang and Semenza, 1995, Semenza, 2003). In hypoxia, the stable HIF-1α translocates to the nucleus and interacts with ARNT, CBP/p300 (cyclic adenosine monophosphate (cAMP)-response-element-binding protein (CREB) binding protein) and DNA Polymerase II, then binds to the conserved *cis*-regulatory site (5'(A/G)CGTG-3') of the HREs of target genes to activate transcription (Lando et al., 2002b, Jiang et al., 1996, Li et al., 1996, Semenza, 1998).

Under normoxic conditions HIF-1α is hydroxylated by iron and 2-oxoglutarate (2-OG) dependent prolyl hydroxylase domains 1, 2 and 3 (PHD1, PHD2 and PHD3; also known as Egln 2, 1 and 3) (Bruick and McKnight, 2001, Epstein et al., 2001). The PHD2 is the most abundant enzyme and plays a dominant role in HIF regulation (Appelhoff et al., 2004). The trans-4-hydroxylation by PHDs occurs at specific proline residues (Pro-402 and Pro-564 in human HIF-1α also, Pro-405 and Pro-531

in human HIF-2α) on the human HIF-1α oxygen dependent degradation domain (ODD) (Jaakkola et al., 2001, Ivan et al., 2001, Masson et al., 2001).

The von Hippel Lindau protein (pVHL) which is an essential component of the E3 ubiquitin ligase binds to the hydroxylated proline residues in HIF-α and facilitates ubiquitination followed by rapid proteasomal degradation (Maxwell et al., 1999). In the absence of pVHL, due to mutations in the *VHL* gene, HIF-α is not degraded. Therefore HIF-α accumulates and genes with HREs are transcriptionally activated (Maxwell et al., 1999, Cockman et al., 2000). Mutations in the *VHL* gene result in hereditary cancer syndromes such as clear cell renal carcinoma (RCC), phaeochromocytoma and hemangioblastoma (Kim and Kaelin, 2004).

Further oxygen regulated control is facilitated by factor inhibiting HIF-1 (FIH-1) (also known as the HIF asparaginyl hydroxylase). FIH-1 hydroxylates the asparagine residue (Asn-803 in human HIF-1α) located in the C-terminal transactivation domain of HIF-1α and prevents binding of the HIF-1α to the transcriptional co-activator p300CBP, thus reducing the transcriptional activity of HIF-1α (Lando et al., 2002a, Mahon et al., 2001).

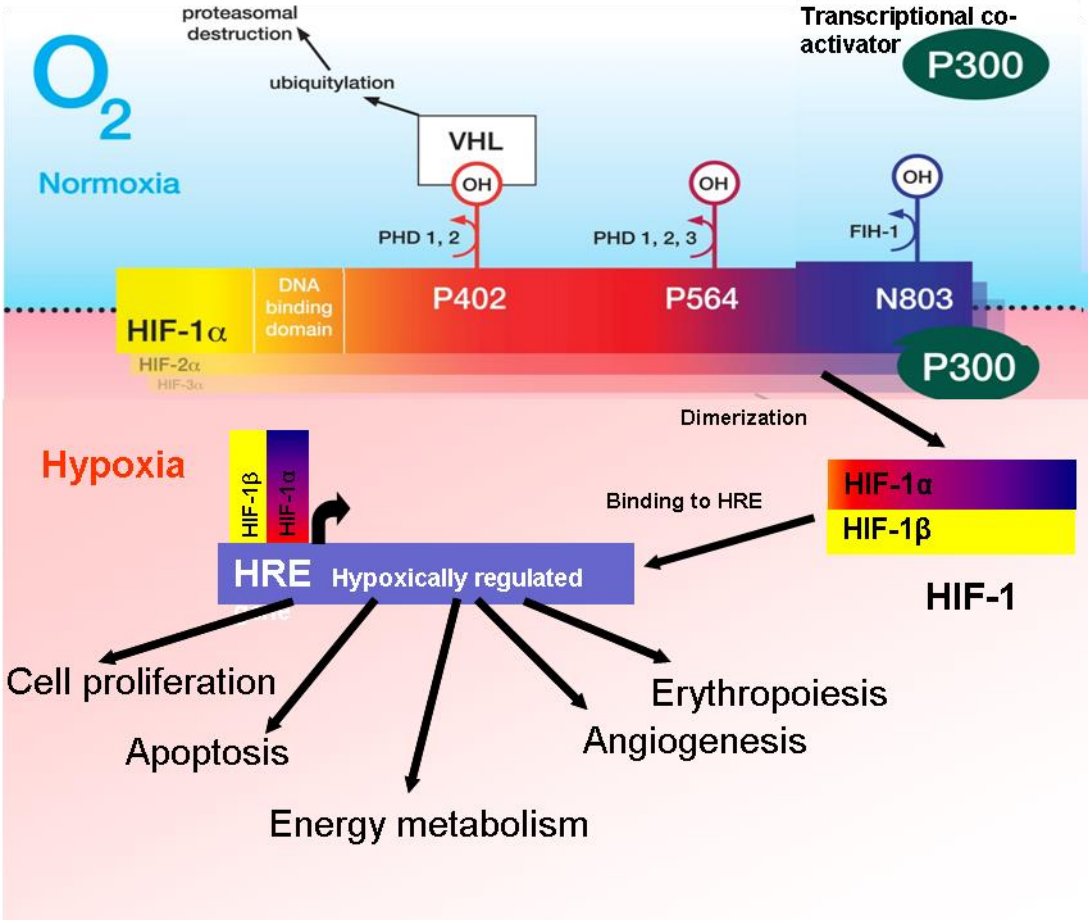

Figure 1.1 Oxygen dependent gene regulation by hypoxia inducible factor-1α (HIF-1α).

Under normoxia, HIF-1α is hydroxylated by PHD enzymes and degraded via pVHL mediated ubiquitin–proteasome pathway. Under hypoxia, HIF-1α is dimerized with HIF-1β and activates transcription of several cellular processes (Reproduced following ((Gleadle, 2009))

The transcription factor HIF-1 plays a dominant role in transcriptional gene regulation in hypoxia (Semenza et al., 1991, Semenza, 2003, Semenza, 2000, Rey and Semenza, 2010). Gene expression arrays examining genome wide transcript analysis demonstrate that hundreds of genes are regulated either positively or negatively by the HIF pathway (Manalo et al., 2005, Elvidge et al., 2006, Hu et al., 2003, Choi et al., 2008). A microarray study examining global changes in gene expression in MCF7 cells in response to hypoxia, dimethyloxalyl glycine (DMOG) and HIF-1α or HIF-2α inhibition by short interfering RNA (siRNAs), showed that a large number of genes were similarly regulated by hypoxia and DMOG (Elvidge et al., 2006). DMOG is a competitive inhibitor of prolyl hydroxylases that stabilizes HIF-α expression at normal oxygen tensions. Most of the genes induced by hypoxia or DMOG were suppressed by the HIF-1α or HIF-2α siRNA, with the majority showing HIF-1α dependence. These results confirmed the significance of gene regulation by the HIF-1 transcriptional system.

Chromatin immunoprecipitation (ChIP) techniques have been used to identify genes directly regulated by HIF binding to DNA (Elvidge et al., 2006, Choi et al., 2008, Ortiz-Barahona et al., 2010, Schodel et al., 2011, Mole et al., 2009). Genome wide analysis of HIF-1α and HIF-2α DNA binding in MCF7 cells using ChIP methods showed the distribution of HIF binding sites across approximately 25,000 human genome promoters (Mole et al., 2009). They also reported a striking difference between the two alpha subunits in gene regulation. Even though many loci could bind to both isoforms, HIF-1α played a more dominant role than HIF-2α in the HIF pathway (Mole et al., 2009). Another study using ChIP coupled with next generation high throughput sequencing (ChIP-Seq) also identified a large number of HIF

binding sites across the genome (Schodel et al., 2011), further demonstrating the extensive regulation by the HIF pathway.

In addition to mediating direct transcriptional effects via HREs, HIF-α also interacts with other regulatory pathways. These include interactions with miRNA networks, such as miR-210 mediated gene regulation in hypoxia (Kulshreshtha et al., 2007a, Kulshreshtha et al., 2007b). HIF-α also interacts with other signalling pathways such as Notch, Wnt and Myc (Kaidi et al., 2007, Gustafsson et al., 2005, Koshiji et al., 2004) and also mediates selective mRNA translation during hypoxia (Uniacke et al., 2012).

1.3.2 HIF independent pathways

Even though majority of the transcriptional regulation in response to hypoxia is mediated through HIF, there are a number of HIF independent pathways that are involved in regulating cellular functions in hypoxia. The following processes show hypoxic regulation and may contribute to the effects of hypoxia on transcriptional gene regulation, protein synthesis and inhibition of translation.

A number of transcription factors are involved in hypoxic gene regulation in addition to HIF (reviewed in (Cummins and Taylor, 2005)), examples include: cyclic AMP response element binding protein (CREB) (Taylor et al., 2000), nuclear factor kappa B (NFkB) (Cummins et al., 2006), Activating protein 1 (AP-1) (Karin and Shaulian, 2001, Shaulian and Karin, 2001), early growth response-1 (EGR-1) (Yan et al., 1999), and the metal-transcription factor-1 (Murphy et al., 1999).

The inhibition of protein synthesis in hypoxic conditions is also mediated through HIF independent pathways. The mammalian target of rapamycin (mTOR),

endoplasmic reticulum kinase (PERK) and AMP- activated protein kinase (AMPK) have been identified to contribute to HIF independent regulation of protein translation in hypoxia (Liu et al., 2006). In chronic hypoxia, AMPK is activated, and phosphorylation and inactivation of eEF2 is increased, resulting in reduced protein translation (by pausing translation temporarily at elongation) (Horman et al., 2002). Another recent study demonstrated that eEF2 phosphorylation and inhibition of protein translation occurred rapidly in acute hypoxia without ATP depletion or AMP kinase activation and was mediated through PHD2 activity in a HIF independent manner (Romero-Ruiz et al., 2012).

Angiogenesis in hypoxia is also regulated through HIF-1 independent mechanisms (Mizukami et al., 2007): by the angiogenic factor IL-8 (interleukin 8) (Mizukami et al., 2005); oncogenic Ras (K-Ras and H-Ras) (Mizukami et al., 2004, Zhang et al., 2001); COX-2 (cyclooxygenase-2) (Pichiule et al., 2004) and transcription factor NF-κB (Mizukami et al., 2005).

1.3.3 HIF expression in cancer

HIF expression and function is enhanced in many cancers (Wong et al., 2011, Semenza, 2000, Mizukami et al., 2007, Zhong et al., 1999). HIF-1 induces or represses transcription of many genes, including genes with key roles in tumour initiation, growth and metastasis (Figure 1.2). HIF induces cell proliferation and reduces cell death by inducing growth or survival factors such as vascular endothelial growth factor (VEGF) (Forsythe et al., 1996), insulin like growth factor-2 (IGF2) (Feldser et al., 1999), endothelin 1 (EDN1) (Grimshaw, 2007) and erythropoietin (EPO) (Semenza and Wang, 1992, Semenza et al., 1991). HIF is also involved in the metabolic reprogramming of cells in hypoxia, by regulating genes encoding glucose

transporters (GLUT1, GLUT2) (Chen et al., 2001) and glycolytic enzymes Aldose A (ALDOA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In hypoxia, cellular transformation takes place by: immortalization through increased telomerase activity via hypoxia regulated mitogen-activated protein kinase (MAPK) signalling (Seimiya et al., 1999); hypoxia induced DNA breaks at fragile sites (Coquelle et al., 1998); and disruption of DNA repair mechanisms (Yuan et al., 2000).

Angiogenesis has a critical role in tumour progression and is driven by hypoxia and HIF-1. In hypoxia, angiogenesis is increased by HIF-1 mediated induction of vascular endothelial growth factor (VEGF) (Shweiki et al., 1992) and platelet derived growth factor B (Rey and Semenza, 2010). HIF-1 also plays a role in epithelial to mesenchymal transition (EMT) by activating repressors (such as Zinc finger E-box binding homeobox1 and 2: ZEB1 and ZEB2) that block the expression of E-cadherin and other proteins involved in maintaining cell to cell adhesion, rigid cytoskeleton and other characteristics of epithelial cells (Esteban et al., 2006). Some genes (lysyl oxidase family (LOX), angiopoietin-like 4 (ANGPTL4) and L1 cell adhesion molecule (L1CAM)) involved in metastasis and invasion are also regulated by HIF-1 (Wong et al., Zhang et al., 2011). Therefore it is apparent that HIFs play important roles in many crucial aspects of cancer biology from tumour initiation to growth and metastasis.

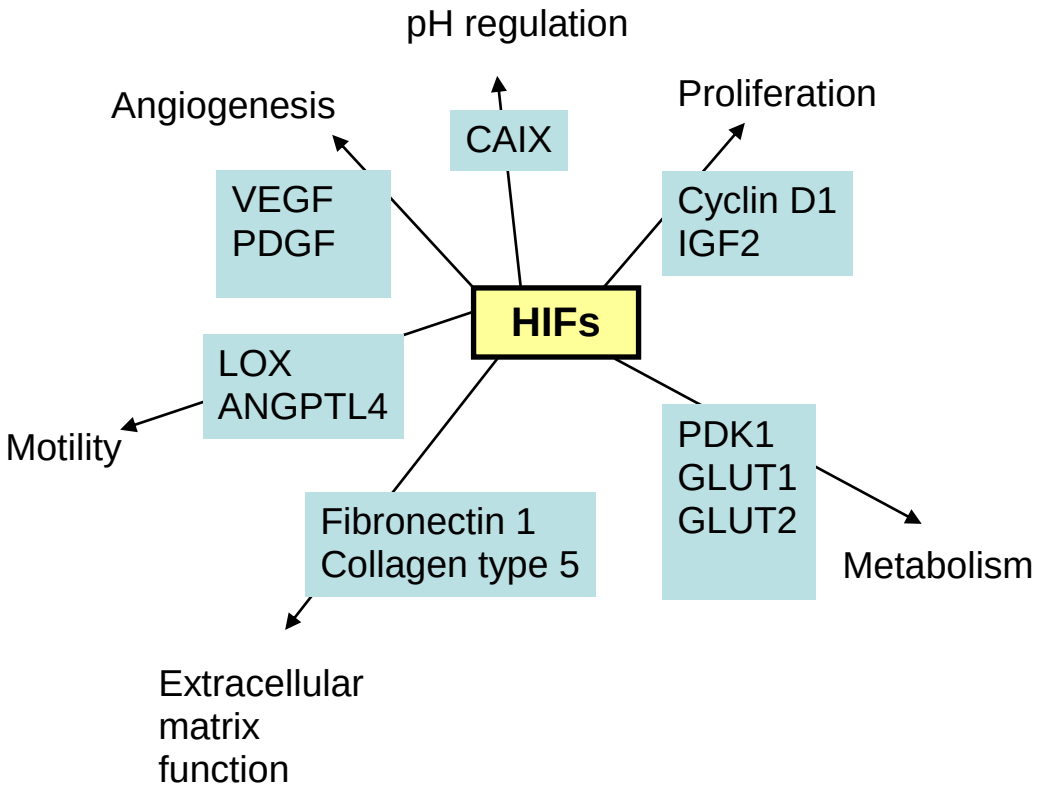

Figure 1.2 Genes activated by hypoxia-inducible factors (HIFs) that are involved in tumour progression.

Genes encoding proteins involved in numerous aspects of tumour initiation, growth and metastasis are transcriptionally activated by either HIF-1α or HIF-2α. carbonic anhydrase IX (CAIX,); C-X-C chemokine receptor type 4 (CXCR4), insulin-like growth factor ii (IGF2,); platelet-derived growth factor B (PDGF); pyruvate dehydrogenase kinase 1 (PDK1); vascular endothelial growth factor A (VEGFA); glucose transporters (GLUT1 and GLUT2); lysyl oxidase family (LOX); angiopoietin-like 4 (ANGPTL4) (Reproduced following (Bertout et al., 2008))

A large number of studies show a correlation between HIF-1α expression, poor prognosis and resistance to therapy in head and neck, ovarian, oesophageal, colon, breast, gastric, lung, skin, pancreatic, prostate and renal carcinomas (Talks et al., 2000, Zhong et al., 1999, Chi et al., 2006). Over expression of HIF-2α correlated with poor prognosis in; non-small cell lung cancer (Giatromanolaki et al., 2001), neuroblastoma (Holmquist-Mengelbier et al., 2006), astrocytoma (Khatua et al., 2003), and head and neck squamous cell carcinomas (Koukourakis et al., 2002). However, in head and neck cancers (Beasley et al., 2002) and non small cell lung cancers (Volm and Koomagi, 2000), HIF-1α over expression was found to correlate with reduced patient mortality. HIF-1α and HIF-2α subunits might be having opposite effects on cancer progression depending on the type of tumour. In patients with VHL disease, HIF-1α expression gradually decreases, while HIF-2α expression increases as renal carcinomas develop (Kaelin, 2007). Studies looking at transcriptional selectivity have shown exclusive HIF-1α targets or HIF-2α targets, and genes that respond to both HIF-1α and HIF-2α (Holmquist-Mengelbier et al., 2006, Hu et al., 2003, Raval et al., 2005). Large differences have been observed in HIF-2α activity in cells that express both HIF-1α and HIF2α, with HIF-2α appearing to be less active than HIF-1α in many cell types (Hu et al., 2006).

1.4 MicroRNAs

MicroRNAs (miRNAs) are 17-22 nucleotide long, non-coding, endogenous, single stranded RNA molecules that play a key role in gene regulation at the posttranscriptional level (Filipowicz et al., 2008, Winter et al., 2009). Functional studies show that miRNAs are involved in regulating many cellular processes including developmental timing, cell differentiation, cell proliferation and cell death (Brennecke et al., 2003, Xu et al., 2003). Furthermore, evidence from many studies shows that the expression of several miRNAs are deregulated in human disease conditions including cancer (Calin et al., 2002, Calin et al., 2004, Deng et al., 2008, Greither et al., 2010, Iorio et al., 2005, Michael et al., 2003). A global reduction in miRNA expression has been observed in both human and mouse cancers when compared to normal tissue (Lu et al., 2005, Volinia et al., 2006, Dvinge et al., 2013).

1.4.1 Discovery of microRNAs

The first miRNA, lin-4 was discovered in 1993 in the nematode worm *Caenorhabiditis elegans*. Lin-4 was found to regulate the *lin-14* gene in *C.elegans* and the expression of lin-4 was essential for the transformation of *C. elegans* from the first to the second larval form (Wightman et al., 1993, Lee et al., 1993). Later, another small RNA molecule let-7 with critical functions in *C.elegans* larval development was discovered (Reinhart et al., 2000, Slack et al., 2000). Since the discovery of the first miRNAs many more have been discovered in plants, animals and humans. Currently, the miRBase repository of published miRNAs contains a total of 2578 human miRNAs miRBase version 20 (miRBase, 2010).

1.5 MicroRNA biogenesis

MicroRNAs are synthesised from larger RNA transcripts by a complex enzymatic pathway. This section will provide a detailed description of the miRNA biogenesis pathway (Figure 1.3). MicroRNAs are transcribed as primary transcripts (pri-miRNAs) (Lee et al., 2004) from independent non-coding RNAs or processed from the introns of protein coding genes miRtrons (Rodriguez et al., 2004). Some miRNAs such as those of the miR-17-22 cluster have been found clustered in polycistronic transcripts (Hayashita, Osada *et al*. 2005). Pri-miRNAs are transcribed by RNA Polymerase II (Lee et al., 2004) or III (Borchert et al., 2006) enzymes, they contain one or several stem-loop structures and can be several kilobases long.

1.5.1 DROSHA processing

DROSHA catalyses the processing of pri-miRNAs into pre-miRNAs in the nucleus. A typical pri-miRNA contains a dsRNA stem (~33 bp) with a terminal loop at one end and flanking ssRNA segments at the other end (Han et al., 2006). Pri-miRNAs are cleaved at the base of the stem loop structure to generate a hairpin called the precursor miRNA (pre-miRNA) (about 70 nucleotides long), by the microprocessor complex formed by the nuclear RNase III enzyme DROSHA (Pasha in *D.melanogaster* and *C.elegans*) and the double stranded RNA-binding protein DiGeorge Syndrome Critical Region gene 8 (*DGCR8*) in humans (Han et al., 2004). A DROSHA processed pre-miRNA contains a (~ 2 nt) 3’ overhang, a dsRNA stem (~ 24 bp long) and a terminal loop. The 3’ end contains a hydroxyl group and the 5’end a phosphate group (Cai et al., 2004).

DROSHA contain two RNase III domains and one double-stranded RNA binding domain (dsRBD). The RNases III domains function as a dimer and cleave the 3' and 5' strands of the hairpin stem (Han et al., 2004). DGCR8 contains two dsRBDs and recognises the pri-miRNA by the single stranded RNA segments flanking the stem loop structure. Then DGCR8 binds to the ssRNA-ds-RNA junction and DROSHA cleaves the pri-miRNA ~11 bp away from this junction (Han et al., 2006).

DGCR8 is a heme binding protein and heme is required for the dimerisation and efficient activation of DGCR8 for pri-miRNA processing (Faller et al., 2007). In a mouse study, it was shown that DROSHA-mediated pri-miRNA processing was regulated by RNA helicases (p72 and p68) bound to the microprocessor complex formed by DROSHA and DGCR8. Both of these RNA helicases were found to be essential for survival of mice and required for the processing of a subset of pri-miRNAs (Fukuda et al., 2007). In addition, arsenite-resistance protein 2 (ARS2) (Sabin et al., 2009, Gruber et al., 2009) and heterogenous nuclear ribonuclear protein (hnRNP A1) (Guil and Caceres, 2007) are also involved in regulating DROSHA mediated processing of specific miRNAs. A component of the RNA cap-binding complex (CBC), ARS2 is important for stability and delivery of some pri-miRNAs to the DROSHA-DGCR8 complex (Gruber et al., 2009). The pri-miR-18a is bound by the hnRNP A1 before DROSHA processing and depletion of hnRNP A1 reduced the abundance of pre-miR-18a levels in HeLa cells (Guil and Caceres, 2007). Another study showed the estrogen receptor-α (ERα) association with p72 and DROSHA under estradiol (E2) stimulation. The association of the ERα-E2 with DROSHA inhibits the binding and processing of some pre-miRNAs, therefore reducing mature miRNA levels (Yamagata et al., 2009). DGCR8 is essential for miRNA biogenesis and loss of DGCR8 correlated with global reductions in mature miRNA expression

(Wang et al., 2007, Yi et al., 2009). Two separate microarray analyses looking at wild type and DGCR8 knockout mouse embryonic stem cells and mouse skin cells, showed a global decrease in mature miRNA expression in the DGCR8 knockout cells (Wang et al., 2007, Yi et al., 2009).

DROSHA-DGCR8 processing is essential for a majority of the miRNAs but there are a small group of miRNAs that can bypass this step. These are derived from short intronic hairpins (miRtrons) and are processed by splicing machinery into pre-miRNA like hairpins, and then processed as a regular pre-miRNA by DICER (Okamura et al., 2007). In addition to miRNA processing, DROSHA-DGCR8 microprocessor complex is also involved in processing hairpin structures from protein coding genes.

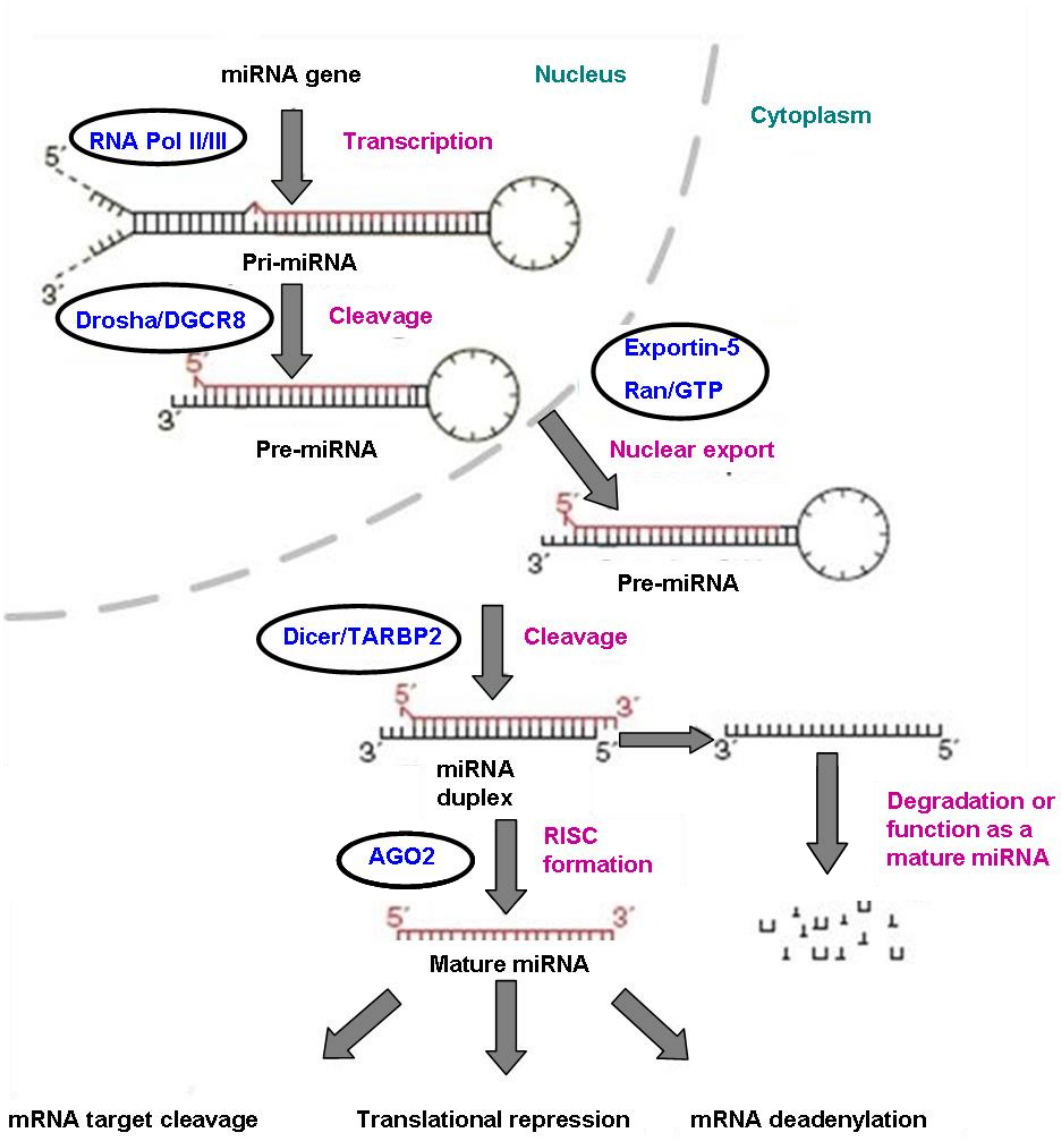

Figure 1.3 Schematic diagram of the canonical miRNA biogenesis pathway.

MicroRNAs are transcribed as primary transcripts (pri-miRNAs) by RNA Polymerase II or III enzymes. In the nucleus, pri-miRNAs are cleaved by micro-processor complex DROSHA and DGCR8 to generate a precursor miRNA (pre-miRNA). Pre-miRNAs are exported to the cytoplasm by exportin-5. In the cytoplasm pre-miRNAs are further cleaved by DICER coupled with TARBP2 to generate a 22 nucleotide double stranded miRNA duplex. One strand of the RNA duplex functions as the mature miRNA and often the passenger strand will be degraded by endonucleases. Mature miRNA binds to RNA induced silencing complex containing an Argonaute protein leading to either translational inhibition or destruction of the target mRNA. (Reproduced following (Winter et al., 2009))

1.5.2 Nuclear export by Exportin-5

Pre-miRNAs are recognised and exported to the cytoplasm by the karyopherin exportin-5 (XPO5), a member of the nuclear transport receptors (Kim, 2004, Bennasser et al., 2011). XPO5 binds to the correctly processed pre-miRNA (with a 3' overhang) in a Ran guanosine triphosphate (Ran-GTP) dependent manner in the nucleus and translocates to the cytoplasm, where hydrolysis of GTP (guanosine triphosphate) to GDP (guanosine diphosphate) releases the pre-miRNA from the complex (Kim, 2004).

XPO5 mediated transport is important for miRNA biogenesis, as depletion of XPO5 reduced mature miRNA levels in the cytoplasm (Yi et al., 2003). However, pre-miRNAs did not accumulate in the nucleus after depletion of XPO5, suggesting pre-miRNAs might be relatively unstable and interaction with XPO5 may be involved in stabilising the pre-miRNAs (Yi et al., 2003). Another study also showed that changes in XPO5 levels affected mature miRNA levels in mouse embryonic fibroblasts (MEF) during cell proliferation, where mature miRNA levels significantly decreased with XPO5 inhibition (Iwasaki et al., 2013). During cell cycle entry, XPO5 levels significantly increased in MEFs compared to other important miRNA biogenesis proteins (such as DROSHA, DGCR8, DICER and AGO2). XPO5 protein levels were found to be highly induced in actively proliferating cells, however, no change in mRNA levels of *XPO5* was observed. They identified that this increase in XPO5 protein levels during cell cycle entry was mediated through a phosphoinositide 3-kinase (PI3K) dependent post-transcriptional mechanism: however, further investigations are needed to understand the exact details of this regulation (Iwasaki et al., 2013).

1.5.3 DICER processing

DICER catalyses the processing of a pre-miRNA into a mature miRNA duplex. In the cytoplasm, pre-miRNAs are further cleaved by DICER ribonuclease III enzyme (Grishok et al., 2001, Hutvagner et al., 2001), coupled with Tar RNA binding protein 2 (TARBP2) (Chendrimada et al., 2005, Haase et al., 2005). This results in a 22 nucleotide double stranded miRNA duplex with protruding 2 nucleotide 3' ends.

DICER is a multi domain protein, with two RNAse III domains, a dsRBD, a long N-terminal containing a DEAD-BOX RNA helicase domain, a Domain of Unknown Function 283 (DUF283) domain and a Piwi Argonaut and Zwille (PAZ) domain. The PAZ domain within DICER binds to the 2 nt 3'overhang and the dsRBD binds to the double stranded stem of small RNAs (MacRae et al., 2007). DICER cleaves the pre-miRNA near the base of the loop 22 nt away from the 3' end.

DICER cleavage is essential for miRNA processing and deletion of DICER decreases mature miRNA expression (Grishok et al., 2001, Hutvagner et al., 2001). Deletion of DICER has been found to cause lethality during early development of mice (Bernstein et al., 2003). In addition, loss of DICER has resulted in defects in normal skeletal muscle development (O'Rourke et al., 2007) and severe dilated cardiomyopathy and heart failure in mice (Chen et al., 2008). However, there are exceptions where DICER independent miRNA biogenesis has been described for a small number of miRNAs (Cifuentes et al., 2010, Langenberger et al., 2012).

The first identified DICER independent miRNA was miR-451, which used AGO2 catalytic activity for processing (Cifuentes et al., 2010, Cheloufi et al., 2010). Pri-

miR-451 is normally processed by DROSHA into a pre-miR-451. This pre-miR-451 differs from other "canonical" miRNAs; as it is encoded in a conserved 42 nt hairpin with a 17 nt stem (Cheloufi et al., 2010), while efficient DICER processing requires a 19 nt stem (Siolas et al., 2005). In addition, the last 6 nt of the 23 nt mature miR-451 extends over the loop region into the complementary strand of the hairpin precursor. The DROSHA processed pre-miR-451 is loaded on to AGO2 and cleaved by AGO2 catalytic centre into an intermediate 30 nt with a 3' end. This intermediate product undergoes polyuridylation and further processed into a 20 nt mature miRNA by a cellular nuclease (Cifuentes et al., 2010). Langenberger et al (2012) suggested ten additional miRNAs (miR-30a, miR-143, miR-374, miR-379, miR-381, miR-134, miR-4417, miR-4516, miR-3676, miR-125b-2) as DICER independent. Six of these miRNAs (miR-30a, miR-143, miR-374, miR-379, miR-381, miR-134) derive from precursor hairpins. Out of these, miR-30a, miR-374a and miR-125b-2 were good DICER substrates, but did not change in response to DICER knockdown (Langenberger et al., 2012).

TARBP2 plays an important role in miRNA biogenesis by being a vital component of the DICER containing complex (Melo et al., 2009, Haase et al., 2005). Depletion of TARBP2 resulted in a decrease in miRNA biogenesis (Melo et al., 2009). Biochemical analysis has revealed the association of TARBP2 with DICER and AGO2 in the RNA induced silencing complex (RISC) loading complex (RLC) formation (Liu et al., 2004, Meister et al., 2004). Furthermore, inhibition of DICER and TARBP2 using siRNAs showed reduced RISC mediated reporter gene silencing (Chendrimada et al., 2005). Therefore, TARBP2 plays an important role not only in miRNA processing, but also in RISC mediated gene silencing. Due to mutations in *TARBP2*, protein level of TARBP2 was reduced and this affected DICER function

and miRNA processing in colorectal cancers (Melo et al., 2009). Re-introduction of TARBP2 into deficient cells restored DICER processing and mature miRNA production (Melo et al., 2009).

Melo et al. (2011) showed that binding of small molecule enoxacin (a fluoroquinolone used as an antibacterial compound) to TARBP2 protein enhances the miRNA biogenesis process. They reported a global increase in miRNA production in colon carcinoma RKO cell line after treatment with enoxacin, and the majority of the up regulated miRNAs had tumour suppressor features (Melo et al., 2011). Treatment with enoxacin had a cancer specific growth inhibitory effect in human cell cultures and xenografted, orthotopic and metastatic mouse models (Melo et al., 2011).

After DICER processing, the double stranded duplex is loaded onto AGO proteins with the aid of Hsp70 and Hsp 90 (heat shock proteins) (Iwasaki et al., 2010). According to the current model, the loading of a small RNA duplex onto the AGO protein requires ATP hydrolysis (Yoda et al., 2009, Kawamata et al., 2009). Subsequently the duplex is unwound in an ATP independent manner (Kawamata et al., 2009). One strand of the RNA duplex is retained by AGO protein and functions as the mature miRNA (or guide strand), while the passenger strand is often degraded by endonucleases (Khvorova et al., 2003, Schwarz et al., 2003). The selection of a single strand from the small RNA duplex depends on the thermodynamical stability of the first 1-4 bases at each end of the RNA duplex, and the strand with the less stable 5’ end is retained as the guide strand (Khvorova et al., 2003, Schwarz et al., 2003). The seed sequences (from 2-8 nt) of mature miRNAs are highly conserved

and these sequences specifically bind to the complementary sequences in the 3'UTR of target mRNAs (Brennecke et al., 2005).

In some instances, the passenger strand (previously referred to as the star form) is not degraded and may function as a mature miRNA in addition to its corresponding miRNA (Lim et al., 2003, Zhou et al., 2010). In *C. elegans* antisense miRNA (passenger strand) have been recovered but at a much lower frequency than the corresponding mature miRNA (Lim et al., 2003). Zhou et al. (2010) found that miR-155-5p and miR-155-3p were highly induced in human plasmacytoid dendritic cells, and they both functioned as mature miRNAs exerting opposite effects on type I interferon production.

1.6 Relationships between biogenesis proteins

The co-ordinated availability of the proteins with roles in miRNA biogenesis is vital to achieve optimal generation of regulatory mature miRNAs. However, the mechanisms underlying this co-ordinate regulation are incompletely understood.

A relationship has been observed between miRNA biogenesis proteins DROSHA and DGCR8 (Han et al., 2009). DROSHA and DGCR8 form the microprocessor complex, and regulate each other post-transcriptionally (Han et al., 2009, Han et al., 2004). The DROSHA-DGCR8 complex cleaves the hairpin structures in the DGCR8 mRNA, reducing DGCR8 mRNA levels. The protein-protein interaction between DGCR8 and DROSHA stabilises the DROSHA protein (Han et al., 2009).

Inhibition of XPO5 increased nuclear localisation of DICER mRNA and down regulated DICER protein levels in the cytoplasm. Post-transcriptional interaction of

XPO5 and *DICER* mRNA has been reported, and an increase in pre-miRNA or adenoviral associated 1 (VA1) RNA resulted in a reduction of this interaction and decreased DICER protein levels (Bennasser et al., 2011). Otherwise XPO5 is not involved in mRNA transport and only known to transport transfer RNAs (tRNAs), non-coding cytoplasmic localized RNAs (Y-RNAs) and pre-miRNAs (Bennasser et al., 2011).

Conflicting results have been reported about the TARBP2 and DICER interaction. Chendrimada et al. (2005) observed that TARBP2 is involved in stabilising DICER and knock down of TARBP2 reduced DICER levels and vice versa. However, another study reported that deletion of TARBP2 had no effect on DICER levels (Haase et al., 2005).

1.7 RNA induced silencing complex

MicroRNAs exert their functional role as part of the RNA induced silencing complex (RISC) (Gregory et al., 2005). The functional mature miRNA strand is loaded into the RISC composed of Argonaute-2 protein (AGO2) bound with a glycine tryptophan repeat containing protein of 182 kDa (GW182) (Eulalio et al., 2008b). The RISC loading complex (RLC), which is composed of DICER, TARBP2, PACT (protein activator of PKR) assist with the loading of the mature miRNA onto the RISC (Gregory et al., 2005, Hammond et al., 2000, Chendrimada et al., 2005, Fabian and Sonenberg, 2012).

In mammals the Argonaute (AGO) family consists of four closely related proteins (AGO1 to AGO4), which seem to have overlapping functions in miRNA mediated

translational repression and cleavage of mRNA through endonuclease activity (Su et al., 2009). When over-expressed, all four AGO proteins seem to bind endogenous miRNAs without selectivity but only AGO2 is able to catalyse cleavage (or possess slicer activity) of miRNAs (Liu et al., 2004, Meister et al., 2004), as it is the only AGO with the RNaseH like P-element induced wimpy testis (PIWI) domain, that allows it to function in RNA interference (RNAi) (Liu et al., 2004). AGO2 proteins are also involved in processing and cleavage of pre-miRNAs in addition to DICER. In some miRNAs, AGO2 cleaves the prospective passenger strand creating a nicked hairpin termed ac-pre-miRNA (Diederichs and Haber, 2007). Loss of endogenous AGO2 seems to reduce steady state levels and activity of mature miRNAs (O'Carroll et al., 2007, Diederichs and Haber, 2007). Mouse embryonic stem cells deficient for AGO1-4 lack miRNA mediated gene regulation, thus the proapoptotic protein BCL2L11 (Bcl-2 like 11) (a miRNA target) is increased, causing cells to undergo apoptosis (Su et al., 2009).

1.8 Gene regulation by microRNAs

Mature miRNAs loaded onto the RISC complex are involved in gene regulation through translational repression and/or mRNA degradation. Some studies suggest repression at translational initiation (Pillai et al., 2005) while others suggest inhibition of post initiation processes (Filipowicz et al., 2008). Generally, in animals, miRNAs post transcriptionally regulate protein synthesis by base pairng to partly complementary sequences in the 3’ untranslated regions (UTRs) of specific mRNAs (Bartel, 2009, Fabian et al., 2010, Krol et al., 2010). Effective miRNA mediated regulation requires the binding of miRNA nucleotides 2 – 8 (seed region) with the target mRNA. Messenger RNA degradation takes place when miRNAs with a high

degree of sequence complementarity bind with the target mRNA, which is then degraded by AGO2 protein cleavage activity (Wahid et al., 2010). Without its interacting partner GW182, AGO proteins fail to silence mRNA targets (Eulalio et al., 2008b). Depletion of GW182 reduced miRNA mediated silencing of reporter mRNAs in human and *Drosophila melanogaster* cells.(Yao et al., 2011, Eulalio et al., 2008b).

In addition, deadenylation, decapping and exonucleolytic digestion of mRNA by miRNA mediated processes are also involved in mRNA decay (Eulalio et al., 2008a, Eulalio et al., 2009). Wu et al., (2006) reported of miRNA mediated acceleration of deadenylation in mammalian cells which led to rapid mRNA decay, another distinct mechanism of post-translational gene regulation. They used miR-125b and let-7 as representative miRNAs, and these miRNAs increased deadenylation of mRNA with imperfect complementarity, thereby accelerating degradation (Wu et al., 2006). A recent study looking at miRNA mediated gene silencing in Drosophila S2 cells showed inhibition of translation and subsequent mRNA deadenulation and decay (Djuranovic et al., 2012).

Some studies argue that miRNA mediated silencing occurs independent of a deadenylation event, as mRNAs lacking polyadenylated tails were also repressed (Pillai et al., 2005, Iwasaki et al., 2009, Eulalio et al., 2009). Other studies argue that miRNA regulated mRNA decay is independent of translation as mRNA decay by this method happened even when translation was disrupted (Eulalio et al., 2009, Zdanowicz et al., 2009, Wu et al., 2006). Guo et al. (2010) has shown that reduced protein output in miRNA mediated gene regulation is mainly due to destabilization of target mRNAs.

In the recent years the understanding of miRNA mediated gene silencing have advanced significantly; however, the exact mechanism of translational repression has not been determined. Possible models have been proposed based on current evidence, with the latest model suggesting inhibition of translation initiation leading to deadenylationm and mRNA decay (Djuranovic et al., 2011).

1.9 MicroRNAs and cancer

Several lines of evidence have suggested an association both with specific miRNAs and general levels of miRNAs and cancer. The connection between miRNAs and cancer was first identified in B-cell chronic lymphocytic leukaemia (CLL), where genes for miR-15 and miR-16 were frequently deleted (Calin et al., 2002). Michael et al. (2003) identified reduced levels of mature miRNAs miR-143 and miR-145 in colon adenocarcinomas (Michael et al., 2003). Since then, many miRNAs have been associated with tumourigenesis. In cancers, different miRNAs were found to be induced or repressed when compared to normal tissue (See review by (Lee and Dutta, 2009).

A study looking at miRNA expression levels in 334 samples, including multiple human cancers observed an overall reduction in mature miRNAs in human cancers when compared to normal tissue (Lu et al., 2005). Rosenfeld et al (2008) showed that miRNA expression analysis can be used to link metastatic cancers with its primary site. The over expression of specific miRNAs, such as miR-21, in human brain tumour (glioblastoma) contributed to its malignant progression. Inhibiting this miRNA induced critical apoptotic pathways in human glioblastomas (Chan et al., 2005, Si et al., 2007). In some instances miRNA expression profiles can be used as

diagnostic and prognostic markers, such as in human lung cancers, where high miR-155 and low let-7a-2 expression correlated with poor survival (Yanaihara et al., 2006). Therefore, these observations suggests a relationship between miRNA expression patterns and tumour progression, metastasis and prognosis in some cancers (Lu et al., 2005, Rosenfeld et al., 2008, Chan et al., 2005, Volinia et al., 2006, Yanaihara et al., 2006). These perturbations in miRNA abundance in cancers have led to the use of miRNA profiling in order to differentiate human cancers from normal tissues and also to classify poorly differentiated tumours (Lu et al., 2005). A miRNA signature for cancer was observed in a study of lung, breast, stomach, prostate, colon and pancreatic tumours, where several cancers associated miRNAs such as miR-17-5p, miR-20a, miR-21, miR-92, miR-106a, *and* miR-155 were consistently up regulated (Volinia et al., 2006).

1.9.1 Specific miRNAs function as oncogenes or tumour suppressors

Different miRNAs can act as oncogenes or tumour suppressor genes. Let-7 acts as a tumour suppressor regulating the oncogenic RAS gene (Johnson et al., 2005). Low let-7 correlates with poor prognosis in lung cancer and head and neck squamous cell carcinomas (Esquela-Kerscher and Slack, 2006). In human and murine colorectal cancers, two miRNAs, miR-143 and miR-145 function as tumour suppressors (Zhu et al., 2010). Epidermal growth factor receptors (EGRF) that contribute to colon tumorigenesis down regulates these two miRNAs, and transfection with these miRNAs inhibited HCT116 colorectal cancer cell growth *in vitro* and *in vivo* (Zhu et al., 2010).

The individual miRNAs with the miR-17-92 cluster function as oncogenes or tumour suppressors by targeting transcription factors E2F and myelocytomatosis oncogene

(MYC). E2F and MYC act as oncogenes or tumour suppressors by regulating cell proliferation or apoptosis depending on their level of expression. The miR-17-92 cluster inhibits E2F and MYC post transcriptionally, while E2F and MYC induce each other and transcription of miR-17-92 cluster, thus creating a negative feedback loop (Aguda et al., 2008).

miR-21 acts as an oncogene by controlling cell growth and inhibiting apoptosis in some cancers; by downregulating antiapoptotic gene *Bcl-2* (B-cell lymphoma 2) (Si et al., 2007, Chan et al., 2005). Studies have shown that miR-21 is up-regulated in human glioblastomas (Chan et al., 2005), breast cancers (Iorio et al., 2005, Yan et al., 2008) and many other solid tumours (Folini et al., 2010, Gao et al., 2010).

1.9.2 MicroRNAs and metastasis

MicroRNAs are also involved in regulating tumour metastasis. Some miRNAs are associated with increased metastasis; while others are involved in suppressing metastasis in cancers (see review (Nicoloso et al., 2009)). miR-10b was highly expressed in metastatic breast cancer cells and has been found to increase cell migration and invasion in human and mouse cells (Ma et al., 2007). In breast cancer, miR-335 and miR-126 have been identified as metastasis suppressors and loss of expression in either of these miRNAs was found to be associated with poor distal metastasis free survival (Tavazoie et al., 2008). miR-31 expression was found to be inversely related to metastasis of human breast cancer and high levels of miR-31 seem to decrease metastasis even in otherwise aggressive breast cancer cells (Valastyan et al., 2009). Recent research has shown that it is possible to use miRNA expression as a molecular marker for metastatic potential. A study of head and neck squamous cell carcinomas (HNSCC) has shown that miR-205 was highly expressed

in metastatic cervical lymph node tissue when compared to non-metastatic tissue. Cervical lymph node metastases in HNSCC could be used for determining patient prognosis. Therefore miR-205 could be used as a molecular marker to detect metastasis in HNSCC and predict prognosis (Fletcher et al., 2008).

1.10 Mechanisms of miRNA dysregulation in human cancer

The mechanisms underlying miRNA dysregulation appear to be a result of several different factors, such as genomic abnormalities, epigenetic factors, transcriptional regulation and altered miRNA processing (See review (Deng et al., 2008)). In addition, most miRNAs are encoded within fragile sites that are often deleted in cancer (Calin et al., 2004).

Some miRNAs are regulated by transcription factors such as p53, which is an important tumour suppressor that acts as a transcriptional regulator affecting the expression of many apoptosis related pathways (Lane and Benchimol, 1990). p53 was found to induce miR-34a expression by binding to a p53 binding site located within the promoter region of the miR-34a gene. Over-expression of miR-34a increases apoptosis, while inactivation of miR-34a decreased apoptosis. Therefore, miR-34a is a direct transcriptional target of p53 that contributes to p53 mediated apoptosis (Raver-Shapira et al., 2007). In addition, it has been shown that p53 activity affects post transcriptional maturation of some miRNAs (miR-16, miR-143 and miR-145) involved in growth suppression by disrupting the function of miRNA biogenesis protein DROSHA (Suzuki et al., 2009). p53 interacts with the DROSHA processing complex and facilitates the processing of pri-miRNA to pre-miRNAs, but

p53 mutants interfere with the association of DROSHA microprocessor complex and DEAD-box helicase p68, decreasing miRNA processing (Suzuki et al., 2009).

The repression of miRNA maturation promotes cellular transformation and tumourigenesis (Kumar et al., 2007), and may be mediated through alterations in miRNA processing enzymes. The ratio of pre-miRNA to mature miRNA seems to increase in some cancer cells, may be due to a problem in miRNA processing (Michael et al., 2003). Michael et al. (2003) observed a decrease in mature miRNAs miR-143 and miR-145 in precancerous and neoplastic colorectal tissues, even though constant levels of unprocessed precursor miRNAs were present in these tissues (Michael et al., 2003). Defects in DROSHA processing have been observed in cancer cells in a mouse study, where primary transcripts were present in high levels but they were not further processed by the enzyme DROSHA (Thomson et al., 2006). Kumar et al. (2007) used short hairpin RNA (shRNAs) to knockdown mRNA levels of the three main regulators of miRNA processing; DROSHA, DGCR8 and DICER1, and observed an increase in cellular transformation and tumourigenesis when compared to controls (Kumar et al., 2007).

Furthermore, decreased levels of DICER and DROSHA have been observed in tumours. Merritt, et al. (2008) reported a correlation between DICER and DROSHA mRNA levels in ovarian cancer cells and patient outcome. They observed low DICER expression was significantly associated with advanced tumour stage and high DICER expression was associated with increased median survival (Merritt et al., 2008). Decreased expression of DICER has been associated with decreased survival in patients with non-small cell lung cancer (Karube et al., 2005, Chiosea et al., 2007). In contrast, no significant association was observed between increased or decreased DICER expression and survival in esophageal cancer patients (Sugito et al., 2006).

DICER1 is a haploinsufficient tumour suppressor in human cancers and loss of one DICER1 allele is sufficient for the formation of tumours in breast, kidney, stomach, intestine, liver, lungs and pancreas (Kumar et al., 2009). Kumar et al. 2009 showed that deletion of one copy of the DICER1 gene reduced survival in a mouse model (Kumar et al., 2009).

1.11 Hypoxic regulation of miRNAs in cancer

MicroRNA expression profiles have been analysed in hypoxia, in different cell types and disease conditions. A small number of miRNAs were induced while others were repressed under hypoxic conditions, and these were referred to as hypoxically regulated miRNAs (HRMs) (Kulshreshtha et al., 2007b, Kulshreshtha et al., 2007a). HRM profiles seem to vary between studies, mainly due to differences in detection methods, exposure time, severity of hypoxia, and the differences in cell types.

Kulshreshtha et al. (2007) identified a group of miRNAs that are induced by hypoxia in colon and breast cancer cell lines. Some of these HRMs (miR-21, 23, 24, 26, 27, 103, 107, 181, 210, and 213) were induced across different cell lines (Kulshreshtha et al., 2007a, Kulshreshtha et al., 2007b). Hua et al. (2006) also identified several miRNAs that were induced by hypoxia and desferrioxamine (an iron chelator that induces HIF-1α expression) treatment in human nasopharyngeal carcinoma epithelioid cell line (Hua et al., 2006). Both studies showed that miR-210 and miR-181 were induced in hypoxia.

Most hypoxically regulated miRNAs were also over expressed in human cancers and associated with poor prognosis (Camps et al., 2008, Greither et al., 2010).

Hypoxically regulated miR-210 has been detected in many tumours with poor prognosis and metastatic potential (Foekens et al., 2008, Camps et al., 2008). Another study showed that miR-210 and miR-155, both hypoxically regulated miRNAs, were associated with poor outcome in pancreatic adenocarcinomas (Greither et al., 2010).

1.11.1 Expression and function of miR-210 in hypoxia

miR-210 was consistently induced in hypoxia across many cancer cell lines including breast, lung, colon, pancreatic, renal and head and neck (Camps et al., 2008, Huang et al., 2009). miR-210 is involved in regulating several processes affected by hypoxia including cell cycle regulation, angiogenesis, DNA damage repair, mitochondrial metabolism and carcinogenesis (see review by (Huang et al., 2010)). Several studies show that miR-210 is up-regulated in cancers (Camps et al., 2008, Greither et al., 2010) and in other physiological conditions that result in tissue hypoxia such as high altitude, anaemia, and inadequate blood supply due to poor vasculature (Pineles et al., 2007, Huang et al., 2010). Over expression of miR-210 in breast cancers correlated with metastasis and poor prognosis (Camps et al., 2008, Foekens et al., 2008, Zhang et al., 2009). Interestingly, in head and neck or pancreatic tumours ectopically implanted into immunodeficient mice, miR-210 expression was linked with repression of tumour initiation and growth (Huang et al., 2009). In a sample of ovarian cancer patients, a high frequency of miR-210 gene deletions and reduced miR-210 expression was observed (Giannakakis et al., 2008).

miR-210 is directly regulated by HIF-1α, but not HIF-2α, through a hypoxia responsive element (HRE) in its promoter region (Huang et al., 2009, Crosby et al., 2009, Camps et al., 2008). Another study also showed miR-210 as a VHL dependent

miRNA in renal cancer, and that it was up-regulated in RCC4 cells with mutated VHL (Neal et al., 2010).

The downstream targets of miR-210 are increasingly being defined. Hypoxia induced miR-210 inhibits Max binding protein (MNT), a transcriptional repressor and a known MYC antagonist, thereby suppressing hypoxia induced cell cycle arrest in many cancer cell lines (Zhang et al., 2009). Crosby et al. (2009) found that hypoxically regulated miR-210 and miR-373 are involved in regulating DNA repair through suppression of RADiation sensitive 52 (RAD52), which is a key component of the DNA repair pathway, and this effect could be partially reversed by antisense inhibition of miR-210 and miR-373 (Crosby et al., 2009). miR-210 also regulates an iron-sulphur cluster assembly protein (ISCU1/2) involved in electron transport and mitochondrial oxidation reduction reactions (Rouault and Tong, 2008). An inverse correlation was seen between miR-210 expression and ISCU1/2 expression, consistent with it being a miR-210 target (Neal et al., 2010, Rouault and Tong, 2008).

1.11.2 Mechanisms involved in hypoxic regulation of miRNA expression

1.11.2.1 miRNAs regulated by transcription factors

The expression of miRNAs under hypoxia may be controlled by hypoxically regulated transcription factors. Some miRNAs are products of genes that are regulated by transcription factors expressed in hypoxic conditions, such as; c-MYC (O'Donnell et al., 2005), p53 (Yan et al., 2009) and HIF-1 (Camps et al., 2008). HIF-1 is the main transcription factor that is induced in hypoxia, and it regulates many genes including those that produce miRNAs. Kulshreshtha et al. (2007) analysed 25

kb promoter regions of all known and predicted miRNAs using a position weight matrix (PWM) method, and found that 40% of human and 50% of mouse miRNA promoters have one or more predicted HIF binding sites (Kulshreshtha et al., 2007a). Therefore, HIF-1 induces the expression of specific miRNAs including miR 210, through its action as a transcription factor (Crosby et al., 2009).

Hypoxia induces the tumour suppressor p53 (Graeber et al., 1994). Expression of the oncogenic miR-17-92 cluster is reduced in hypoxic conditions in wild-type p53 cells, but unchanged in p53 deficient cells (Yan et al., 2009). When overexpressed, the miR-17-92 cluster inhibited hypoxia mediated apoptosis in cancers: therefore p53 repression of this cluster is important as it acts as a tumour suppressor in hypoxia (Yan et al., 2009). In addition, p53 is also involved in regulating miR-34a expression which is involved in apoptosis (as discussed in section 1.10) (Raver-Shapira et al., 2007). Another transcription factor induced in hypoxia, c-MYC is involved in inducing the miR-17-92 cluster and thus tumour formation (O'Donnell et al., 2005).

1.11.2.2 Regulation of miRNA biogenesis machinery by hypoxia

MicroRNA regulation in hypoxia may also be achieved through regulation of activity or abundance of the miRNA biogenesis machinery. There are several lines of evidence suggesting a possible link between hypoxia and activity of proteins involved in miRNA synthesis and function (Elvidge et al., 2006, Caruso et al., 2010, Fahling et al., 2004, Qi et al., 2008). Microarray studies by Elvidge et al. (2006) showed modest, but consistent hypoxic reductions in mRNA levels encoding some miRNA biogenesis machinery including *DICER*, *AGO2*, DEAD/H box 5 (*DDX5)*, RNA polymerase III subunit C5 (*RPC5)*, polymerase (RNA) III (DNA directed) polypeptide K *(POLR3K)* and Transcription initiation factor TFIID subunit 9B

(*TAF9B)* (Elvidge et al., 2006). A study using rats and rat pulmonary artery fibroblasts exposed to chronic hypoxia showed reduced DICER expression (Caruso et al., 2010). Hydroxylation by collagen prolyl-4-hydroxylase (C-P4H1), an oxygen dependent, hypoxically regulated enzyme (Fahling et al., 2004), stabilises AGO2, while human cells depleted of C-P4H1 showed reduced AGO2 stability. These findings show the importance of hydroxylation for AGO2 stability and effective RNA silencing (Qi et al., 2008). This process is oxygen dependent and regulated by hypoxia: therefore, AGO2 stabilisation may be regulated by hypoxia which may affect gene regulation by miRNA. However, in other experimental settings such regulation has not been seen. Donker et al. (2007) showed that even though hypoxia dysregulated miRNA expression, it did not affect the key miRNA biogenesis proteins (DROSHA, XPO5, DICER and AGO2) or their mRNA levels in human trophoblasts (Donker et al., 2007). The extent and circumstances in which hypoxia influences the expression and function of enzymes central to miRNA generation and function is therefore unclear, particularly in cancer.

1.12 Rationale for this study

The links between tumour hypoxia, miRNA expression and cancer aggression raises the possibility of a general effect of hypoxia on miRNA biogenesis and function. Given the many observations that tumour cell lines and cancers are usually characterised by global reductions in miRNA expression (Lu et al., 2005) when compared to normal tissue, it is important to understand the mechanisms underlying this difference. Several lines of evidence point towards global reduction in miRNA abundance in cancer and also to influences of hypoxia on the enzymes with critical roles in miRNA manufacture. It is possible that the hypoxic regulation of miRNA biogenesis proteins might contribute to the reduction of mature miRNA expression and hypoxia may have a key role in cancer progression through this mechanism.

This thesis explores the role of hypoxia in mediating miRNA abundance, particularly to understand whether alteration in miRNA abundance is due to changes in miRNA biogenesis proteins in hypoxia. It also examines the mechanisms and consequences of such changes and investigates the possibility that hypoxic regulation of miRNA biogenesis proteins might contribute to the reduction in miRNA expression in many tumours and the role of hypoxia in cancer progression.

Chapter 2 . Materials and methods

2.1 Cell culture procedures

2.1.1 Cell lines and culture procedures

All cell lines used in this thesis (Table 2.1) were maintained in polystyrene flasks with vented caps (Greiner). MCF7, SKBR3 and HT29 cells were cultured in RPMI 1640 media (Invitrogen) supplemented with 10% foetal bovine serum (FBS). RCC4+VHL and RCC4-VHL cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% FBS. Primary cell line human umbilical vein endothelial cells (HUVECs) were maintained in polystyrene flasks coated with gelatine in Media 200 PRF (Invitrogen) supplemented with 20% fetal bovin serum (FBS) and 100μg/mL penicillin/streptomycin. All cell lines were maintained in a humidified incubator with 5% CO_2 at 37°C.

The collection of primary human umbilical vein endothelial cells (HUVEC) for use in this study was given ethical clearance from the Royal Adelaide Hospital (RAH), Adelaide, South Australia. Consent was obtained from all subjects in accordance with the 'Declaration of Helsinki' and conforms to the guidelines established by the National Health and Medical Research Council of Australia.

2.1.1.1 Subculturing cells

Adherent cells were washed once with sterile 1x phosphate buffered saline (PBS), and incubated with 1x TriplE Express (Invitrogen) for 5 min at 37°C. Fresh media was added to deactivate TriplE Express and detached cells were re-plated at the desired density.

2.1.1.2 Cell counting

Adherent cells were washed with sterile 1x PBS and incubated with 1x TriplE Express (Invitrogen) for 5 mins at 37°C. Then detached cells were re-suspended in cell culture medium to obtain a fully dissociated cell suspension, and counted using an inverted microscope and a Neubauer ruled haemocytometer.

2.1.1.3 Cryo-preservation of cells

For cryo-preservation, cultured cells were harvested and pelleted by centrifugation at 136 relative centrifugal forces (RCF) for 5 min, and resuspended in basal media containing 10% FCS. The resuspended cells were mixed with 20% FBS and 10% dimethyl sulphoxide (DMSO), aliquoted into cryo-vials and slowly frozen in a special container (Mr Frosty-Thermo Scientific) at -80°C overnight before transferring to long-term storage boxes at -80°C. For resuscitation, cells were thawed rapidly in a 37°C water bath and mixed with pre-warmed medium in a polystyrene flask.

2.1.1.4 Cell viability assay - Trypan blue exclusion method

A 0.4% trypan blue (Life Technologies) solution was prepared in PBS solution (pH 7.2 to 7.3). 0.1 mL of trypan blue stock solution was added to 1 mL of cells. Then loaded onto a hemacytometer and examined immediately under a microscope at low magnification. The number of cells with blue staining and the number of total cells were counted. Cell viability was calculated by dividing the number of viable cells by the total number of cells within the grids on the hemacytometer. If cells take up

trypan blue, they were considered non-viable. Cell viability should be at least 95% for healthy log-phase cultures.

Table 2.1 Human derived cell lines

Cell line	Derivation	Source
MCF7	Mammary gland adenocarcinoma	ATCC, Manassas, VA, USA
SKBR3	Mammary gland adenocarcinoma	ATCC, Manassas, VA, USA
HT29	Colorectal adenocarcinoma	ATCC, Manassas, VA, USA
RCC4 +VHL	VHL deficient Renal cell carcinoma cell line Stably transfected with pcDNA3-VHL. Encodes the von Hippel-Lindau (VHL) tumour suppressor gene product pVHL.	European Collection of Cell Cultures, UK
RCC4 -VHL	VHL deficient Renal cell carcinoma cell line stably transfected with an empty expression vector, pcDNA3	European Collection of Cell Cultures, UK (Maxwell et al., 1999)
HUVECs	Umbilical vein endothelial cells from multiple donors	Claudine Bonder, IMVS, Adelaide, Australia

2.1.2 Exposure of cultured cells to experimental conditions

2.1.2.1 Exposing cells to different O_2 levels

A hypoxic incubator (Coy Laboratory Hypoxic workstation glove box) was used to expose cells to continuous controlled hypoxic conditions. This was a humidified, temperature controlled (37 ºC) chamber supplemented with 5% CO_2, room air and N_2 (as required to maintain controlled O_2 levels). Cells were exposed to 0.1% or 1% O_2 levels for varying time durations. Parallel controls were incubated in normoxic conditions in a humidified incubator supplemented with 5% CO_2 at 37 ºC.

2.1.2.2 Exposing cells to different chemical compounds

Dimethyloxalylglycine (DMOG) (Enzo Life Sciences) was dissolved in sterile water to make a 100 μM stock solution. Cells were treated with a final concentration of 1mM DMOG and incubated at 37 ºC in a normoxic incubator for 48 h.

Similarly Desferrioxamine (DFO) (Sigma-Aldrich) was dissolved in sterile water to make a 100 μM stock solution. Cells were treated with a final concentration of 0.1mM DFO and incubated at 37 ºC in a normoxic incubator for 48 h.

Z-Leu-Leu-Leu-al (MG-132) was dissolved in DMSO to make a 10 mM stock solution. 100 μL of the stock solution was further diluted in serum free RPMI media to make a 1 mM solution and filter sterilised by passing through a 0.25 μm filter. Cells were treated with a final concentration of 10 μM MG-132 and control plates were treated with only DMSO.

2.1.3 Transient transfection of cultured cells

2.1.3.1 Transient transfection with small interfering RNAs (siRNAs), miRNA mimics and miRNA inhibitors

Cells were seeded at 5 x 10^4 cells per well in 24-well plates and grown for 24 h. For each transfection sample siRNA-Lipofectamine 2000 complexes were prepared as follows: siRNAs (see table 2.2 for list of siRNAs) were diluted in 50 μL of Opti-MEM I (Invitrogen) (serum free medium) to make a final concentration of 20 nM in each well. 1 μL of Lipofectamine 2000 regent was diluted in 50 μL of Opti-MEM I, mixed gently and incubated for 5 min at room temperature. After the incubation the diluted siRNA and Lipofectamine 2000 were combined and mixed gently, then incubated for 20 minutes at room temperature. Then siRNA-Lipofectamine 2000 complexes were added to each well containing cells and medium and mixed gently by rocking the plate. The plates were incubated for 24 h and then a second transfection was done following the above procedure to ensure complete knock down of the gene of interest. 24 h after the second transfection cells were harvested or exposed to desired experimental condition (e.g. exposure to hypoxia vs. normoxia)

Table 2.2 List of siRNAs

siRNA	siRNA sequence	Company	Reference
DICER	5’-UCCAGAGCUGCUUCAAGCATT-3’ 5’-UGCUUGAAGCAGCUCUGGATT-3’	Shanghai GenePharma Co., Ltd	(Chu et al., 2010)
DROSHA	5-ACGAAGCUCGAUGAAGAUUUATT-3’ 5’-UAAAUCUUCAUCGAGCUUCGUTT-3’	Shanghai GenePharma Co., Ltd	(Han et al., 2012)
TARBP2	5’-AAUUCACCAUGACCUGUCGAGTT-3’ 5’-CUCGACAGGUCAUGGUGAAUUTT-3’	Shanghai GenePharma Co., Ltd	(Christensen et al., 2007)
HIF-1α	5’-CUGAUGACCAGCAACUUGAdTdT-3’ 5’-UCAAGUUGCUGGUCAUCAGdTdT-3’	Dharmacon, Lafayette, CO, USA	(Sowter et al., 2003)
HIF-2α	5’-CAGCAUCUUUGAUAGCAGUdTdT-3’ 5’-ACUGCUAUCAAAGAUGCUGdTdT-3’	Dharmacon, Lafayette, CO, USA	(Sowter et al., 2003)
PHD1	5’-CUAUAUCGUGCCCUGAUGTT-3’ 5’-CAUGCAGGGCACGAUAUAGUC-3’	Shanghai GenePharma Co., Ltd	(Appelhoff et al., 2004)
PHD2	5’-GGACAUCCGAGGCGAUAAGTT-3’ 5’-CUUAUCGCCUCGGAUGUCCTT-3’	Shanghai GenePharma Co., Ltd	(Appelhoff et al., 2004)
FIH-1	5’-CAGCGGCGGAGGCUGUGGCTT-3’ 5’-GCCACAGCCUCCGCCGCUGTT-3’	Shanghai GenePharma Co., Ltd	(Stolze et al., 2004)
NC	5’-UUCUCCGAACGUGUCACGUTT-3’ 5’-ACGUGACACGUUCGGAGAATT-3’	Shanghai GenePharma Co., Ltd	

2.1.3.2 Transfection with Plasmids

Cells were seeded at 1 x 10^5 cells per well in 24-well plates and grown for 24 h. For each transfection sample plasmid DNA-Lipofectamine 2000 complexes were prepared as follows: 0.5 μg of plasmid DNA (see table 2.3 for list of plasmid constructs) was diluted in 50 μL of Opti-MEM I (Invitrogen) (reduced serum medium without serum). 1 μL of Lipofectamine 2000 regent was diluted in 50 μL of Opti-MEM I, mixed gently and incubated for 5 min at room temperature. After the incubation the diluted plasmid DNA and Lipofectamine 2000 were combined and mixed gently, and incubated for 20 min at room temperature. The DNA-Lipofectamine 2000 complexes were added to each well containing cells and medium, and mixed gently by rocking the plate. The plates were incubated at 37 C for 2 h and then exposed to hypoxia or normoxia for 24 h before harvesting.

The optimal plasmid DNA: Lipofectamine 2000 ratio was determined by transfecting SKBR3 cells with varying amounts of plasmid DNA (containing a green floursence protein (GFP) promoter-MM043) and Lipofectamine 2000 reagent following the above protocol for plasmid transfections. After 24 hours the plates were scanned using the Typhoon 9400 variable mode imager (Amersham Biosciences, Piscataway, NJ, USA) and the best ratio was determined by quantifying the GFP expression.

Table 2.3 List of plasmid constructs

Plasmid	Source/Reference
pEF-BOS-cs-HIF1αP402AP564G contains the full length HIF1α coding sequence bearing P402A and P564G point mutations in pcDNA3 cloned into pEF-BOS	Dr Dan Peet, Adelaide University
pEF-BOS-HIF2αP531AN847A contains the full length HIF1α coding sequence bearing P531A and N847A point mutations in pcDNA3 cloned into pEF-BOS	Dr Dan Peet, Adelaide University
pCMV-miR-200b containing a pre-miR-200b sequence	Origene
pCMV-miR Empty vector without a miRNA sequence	Origene
pCi-neo-ZEB1-hRL containing the 3'UTR from the ZEB1 gene downstream of a Renilla luciferase reporter	(Gregory et al., 2008)
pGL3-control contains SV40 promoter and enhancer sequences, upstream of a firefly luciferase reporter	Promega

2.2 RNA protocols

2.2.1 RNA extraction

Trizol (Invitrogen) reagent was added directly to adherent cells in plates and homogenised by passing the cell lysate several times through a pipette. The amount of Trizol added was based on the area of the culture plate (1 ml per 10 cm^2). The homogenised sample was incubated at 15 ºC to 30 ºC for 5 min to allow complete dissociation of nucleoprotein complexes. After the incubation, 0.2 ml of chloroform per 1 ml of Trizol was added, mixed well by shaking the tube vigorously by hand for 15 seconds and incubated at 15 ºC to 30 ºC for 2-3 min. After the short incubation the sample was centrifuged at 12000 x g for 15 min at 2-8 ºC. Following centrifugation the mixture separates into a lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase containing RNA. The aqueous phase containing RNA was transferred into a new tube and mixed with isopropyl alcohol (0.5 ml per 1 ml of Trizol) to precipitate RNA. The sample was incubated at 15 ºC to 30 ºC for 10 min and centrifuged at 12000 x g for 20 min at 2-8 ºC. Afterwards the supernatant was removed and the RNA pellet was washed with 75% ethanol (1 ml per 1 ml of Trizol) by mixing well. The mix was centrifuged at 7500 x g for 5 min at 2-8 ºC, the supernatant removed and the pellet was allowed to air dry for 5-10 min. Finally the RNA pellet was re-dissolved in nuclease free sterile water by mixing well and RNA samples were stored at -80 ºC.

2.2.2 RNA quantitation

RNA was quantified using the NanoDrop 8000 spectrophotometer (Thermo Scientific). RNA integrity was assessed by agarose gel electrophoresis or by the Agilent 2100 bioanalyser using a RNA 6000 Pico Chip.

2.2.3 Microarray analysis

Total RNA from MCF7 cells exposed to hypoxia or normoxia was extracted using the TRIzol protocol as above. RNA integrity was assessed using the Agilent 2100 Bioanalyzer. Affymetrix miRNA 3.1 Array Strip was used for RNA analysis. This array consisted of probe sets unique to human mature and pre-miRNA hairpins. A detailed protocol can be found in the miRNA 3.1 Array Strips technical manual (Affymetrix). In summary, 100-300 ng of total RNA was used to synthesise double stranded cDNA using random hexamers. The complementary DNA (cDNA) was then amplified to produce antisense cRNA, which was then reverse transcribed in a second cycle of cDNA synthesis. The second cycle incorporates deoxyuridine triphosphate (dUTP) into the cDNA sequence, which allows it to be fragmented using uracil DNA glycosylase and apurinic/apyrimidic endonuclease I. Following biotinylation, these fragments were hybridised overnight to a Affymetrix miRNA 3.1 array. The arrays were then washed, stained using a fluorescent-labelled antibody, and scanned using a high-resolution scanner. Intensity data was analysed using Partek® software (Partek Inc.). Data was normalised by quantile normalisation and log 2 transformed. Differential expression was determined by ANOVA and corrected for false discovery.

2.2.4. Relative quantification of real time RT-PCR for miRNA

For miRNA expression analysis, cDNA was synthesised using total RNA, TaqMan miRNA specific primers and TaqMan miRNA reverse transcription kit (Invitrogen). Each reaction contained 5 ng of total RNA, 1.5 μL of specific TaqMan RT primer and 3.5 μL RT master mix (made up of 0.075 μL 100M dNTP mix, 0.75 μL of 10x RT buffer, 0.5 μL MultiScribe RT enzyme, 0.095 μL of RNase inhibitor and 2.08 μL of nuclease free water). The samples were then loaded on to a thermo cycler (Applied Biosystems) and cDNA was synthesised by incubating at 16 ºC for 30 min, 42 ºC for 30 min, and 85º C for 5 min and at 4 ºC until ready to use.

Quantitative real time polymerase chain reaction (qRT PCR) was done following the TaqMan protocol in triplicate. Each reaction was done in 0.1 mL PCR tubes (Axygen, Fisher Scientific) and contained 1 μL cDNA, 5 μL of TaqMan universal PCR master mix, 0.5 μL of specific TaqMan miRNA real time primer and 3.84 μL of nuclease free water. The tubes were then loaded on to a thermo cycler (Corbert Rotogene 2000 or Qiagen Rotogene Q) and incubated at 95 ºC for 10 min, then 50 cycles; denaturing step at 95 ºC for 15 sec and annealing/extension step at 60 ºC for 60 sec.

The different TaqMan miRNA assays used are listed in table 2.4. Small nuclear RNA RNU6B was used as a control gene. Data was analysed using Corbett Rotogene software (Version 5.0.61) (Corbett Research). The miRNA expression levels were calculated based on Ct values using Qgene software.

2.2.5 Relative quantification of real time RT-PCR for precursor and mature miRNA

For the parallel detection of precursor and mature miRNA expression cDNA was synthesised using total RNA, miScript HiFlex buffer and miScript ii reverse transcription kit (Qiagen). Each reaction contained 1 μg of total RNA, 4 μL of 5x miScript HiFlex buffer, 2 μL miScript nucleics mix, miScript RT master mix and nuclease free water to make up a 20 μL reaction. The samples were then loaded on to a thermo cycler (Applied Biosystems) and cDNA was synthesised by incubating at 37 ºC for 60 min, 95 ºC for 50 min, and then at 4 ºC until ready to use.

Quantitative real time polymerase chain reaction (qRT PCR) was done following the miScript SYBR Green PCR kit with miScript precursor assays or miScript primer assays following the manufacture's protocol in triplicate (see table 2.4 for list of assays).

For detecting pre-miRNA levels, each reaction was done in 0.1 mL PCR tubes (Axygen, Fisher Scientific) and contained 1 μL cDNA (5 ng/ μL), 10 μL of 2x QuantiTech SYBR Green PCR master mix, 2 μL of 10x specific miScript precursor assay and 10 μL of nuclease free water.

For detecting mature miRNA levels, each reaction was done in 0.1 mL PCR tubes (Axygen, Fisher Scientific) and contained 1 μL cDNA (5 ng/ μL), 10 μL of 2x QuantiTech SYBR Green PCR master mix, 2 μL of 10x specific miScript primer assay, 2 μL of 10x miScript universal primer and 6 μL of nuclease free water.

The tubes were loaded on to a thermo cycler (Corbert Rotogene 2000 or Qiagen Rotogene Q) and incubated at 95 ºC for 15 min, then 50 cycles; denaturing step at 94 ºC for 15 sec, annealing/extension step at 55 ºC for 30 sec and 70 ºC for 30 sec.

Small nuclear RNA RNU6B was used as a housekeeping gene. Data was analysed using Corbett Rotogene software (Version 5.0.61) (Corbett Research). The miRNA expression levels were calculated based on Ct values using Qgene software.

2.2.6 Relative quantification of real time RT-PCR for mRNA

For mRNA expression analysis 1μg of total RNA was diluted in 12.5 μL of nuclease free water and DNase treated by adding 1 μL of DNase and 2.5 μL of DNase buffer (New England BioLabs) and incubated at 37 ºC for 20 min. The reaction was stopped by adding the DNase stop reagent or "slurry". The DNase treated RNA (1μg) was incubated with random primer 6 (New England Labs) (100 ng) at 70 ºC for 5 min and on ice for 5 min. To the reaction 1 μL of M-MLV reverse transcriptase RNase H minus, point mutant (Promega), 5 μL of RT buffer, 1.25 μL of dNTP mix and 6.75 μL of nuclease free water was added and mixed gently. The reaction was incubated at room temperature for 10 min and then loaded on a thermal cycler and incubated at 50 ºC for 50 min and 70 ºC for 15 min. Real time PCR was subsequently performed in triplicate with 1:5 dilution of cDNA using TaqMan gene expression assays following the TaqMan gene expression assay protocol (see table 2.5 for mRNA gene expression assays). Each reaction was done in 0.1 mL PCR tubes (Axygen, Fisher Scientific) and contained 1 μL of diluted cDNA, 5 μL of 2x TaqMan Gene Expression master mix, 0.5 μL of specific TaqMan Gene Expression assay mix and 3.5 μL of nuclease free water. The tubes were then loaded on to a thermo cycler (Corbert Rotogene 2000 or Qiagen Rotogene Q) and incubated at 50 ºC for 2 mins

and 95 ºC for 10 min, then 50 cycles; denaturing step at 95 ºC for 15 sec and annealing/extension step at 60 ºC for 60 sec. *β-2-microglobulin* and 18S genes were used as control genes. The data was analysed using Corbett Rotogene software (Version 5.0.61) (Corbett Research). The mRNA expression levels were calculated based on Ct values using Qgene software.

2.2.7 Statistical analysis

All experiments were performed in triplicate. The data represent normalized mean ±S.E (error bars) (n=3). Statistical significance established by two tailed, paired student's t-test using GraphpadPrism software. A P value <0.05 was considered to indicate statistical significance.

Table 2.4 List of pre-miRNA and miRNA assays

miRNA	Assay type	Supplier
has-miR-210	TaqMan miRNA assay	Life Technologies
has-miR-103	TaqMan miRNA assay	Life Technologies
has-miR-107	TaqMan miRNA assay	Life Technologies
RNU6B	TaqMan miRNA assay	Life Technologies
has-miR-21	miScript primer assay	Qiagen
has-miR-185	miScript primer assay	Qiagen
has-let-7a	miScript primer assay	Qiagen
RNU6B	miScript primer assay	Qiagen
Pre-miR-21	miScript precursor assay	Qiagen
Pre-miR-185	miScript precursor assay	Qiagen
Pre-let-7a	miScript precursor assay	Qiagen

Table 2.5 List of mRNA assays

mRNA	Assay type	Supplier
DICER	TaqMan Gene expression assay	Life Technologies
DROSHA	TaqMan Gene expression assay	Life Technologies
CAIX	TaqMan Gene expression assay	Life Technologies
AGO2	TaqMan Gene expression assay	Life Technologies
18S	TaqMan Gene expression assay	Life Technologies
β-2-microglobulin	TaqMan Gene expression assay	Life Technologies
TARBP2	TaqMan Gene expression assay	Life Technologies

2.3 Protein protocols

2.3.1 Protein extraction

2.3.1.1 Preparation of whole cell extracts

Cells in tissue culture plates were washed once in 1xPBS after removing media and lysed by adding 300 µL (for 6-well plates) or 70 µL (for 24-well plates) of protein lysis buffer (6.7 M urea, 10 mM Tris-HCl (pH 6.8), 10% glycerol and 1% SDS) supplemented with 1 mM DTT and Complete Protease Inhibitor Cocktail Tablets (Roche Applied Science, UK) just before use. The wells were scraped with a cell scraper and lysates were transferred to an Eppendorf tube before being vortexed vigorously for 30 sec, passed through a 26 gauge needle and stored at -20 ºC.

2.3.1.2 Protein quantitation

Extracts were assayed for total protein using EZQ protein quantification kit (Life Technologies). A series of standards (ranging from 0.02-2 mg/ml) were prepared by diluting a 2mg/ml ovalbumin solution in protein lysis buffer. Then 1 µL of each standard, protein sample and control samples (lysis buffer only) were spotted in triplicate onto the prepared assay paper. The paper was allowed to air dry, fixed with methanol for 5 mins and dried again before being stained with EZQ protein quantification reagent for 30 mins with gentle agitation and washed (3 x 2 mins) with a de-stainer (10% methanol and 7% Acetic acid). The fluorescence was detected by scanning the assay paper using the Typhoon scanner. Carestream Molecular imaging software was used to analyse the results. Fluorescence values of the standards and samples were calculated by subtracting the fluorescence value of controls. A standard curve was plotted using the corrected fluorescence values of the standards

vs. the corresponding protein mass. The mass of the protein samples was determined using the standard curve.

2.3.2 Immunoblotting

Total protein extracts (30-50 μg) were resolved by using 8% SDS polyacrylamide gel or Mini-PROTEAN TGX stain-free, AnyKD gradient gel (BIO-RAD) electrophoresis and then electro-blotted on to a polyvinylidene difluoride membrane (Millipore).

2.3.2.1 Sample preparation

To each protein sample (e.g. 50 μg in 30 μL), 3xSDS loading buffer (187 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 0.03% bromophenol blue and 125 mM DTT added just before use) (15 μL) was added and then heated at 100 ºC for 5 min before loading on to a gel.

2.3.2.2 Pouring SDS-PAGE gels

The SDS-PAGE gel consisted of a 5 % stacking gel (upper gel) and an 8% separation gel (lower gel). The 8% separation gel was made up of 2.5 mL 4x separation gel buffer (1.5 M Tris-HCl pH 8.8, 0.4% SDS), 2.5 mL 40 % acrylamide/bisacrylamide, 5.5 mL water, 33 μL 10% Ammonium persulphate (APS) and 10 μL of TEMED. Once the separation gel was set the stacking gel was poured and a gel comb was inserted. The 5 % stacking gel was made up of 1.25 mL 4x stacking gel buffer (0.5 M Tris-HCl pH 6.8, 0.4% SDS), 625 μL of 40% acrylamide/bisacrylamide, 33 μL 10% Ammonium persulphate and 10 μL of TEMED.

The gel was placed in a gel tank with 1xSDS running buffer (25 mM Tri Base, 200 mM glycine, 0.1% SDS) then protein samples (prepared as above) and a pre-stained protein marker (7-175 KDa) (New England BioLabs) were loaded on to the gel. The gel was run at a constant current of 25 mA for 45 min.

2.3.2.3 Using pre-cast gels

When Mini-PROTEAN TGX stain free, AnyKD gradient gels (BIO-RAD) were used for electrophoresis, sample preparation and loading was done following above protocol. The gel was placed in a Mini-PROTEAN tetra Cell (BioRad), filled with 1x running buffer (25mM Tris, 192 mM glycine and 0.1% SDS) and run at a constant voltage of 160 V for 38 min. The Mini-PROTEAN TGX stain free gel was imaged with the Chemi-doc imager (BioRad) after electrophoresis to visualise protein loading. Electroblotting followed the same protocol as below. After electroblotting the PVDF membrane was imaged again using the Chemi-Doc system to detect total protein content on the blot.

2.3.2.4 Electro-blotting

Electro-blotting was performed using a fully submerged transfer apparatus (Mini Trans-blot BioRad system). Transfer buffer consisted of 10 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) and 5% methanol (pH 11 with NaOH). After electrophoresis the gel was soaked in transfer buffer for 30 mins, and then assembled into a transfer sandwich in a transfer cassette in the following order; sponge, 2 layers of filter paper, gel, polyvinylidene difluoride (PVDF) membrane (Millipore), 2 layers of filter paper and another sponge. The direction of protein transfer was from (–) to (+).

Once the cassettes were assembled and inserted, the tank was filled with ice cold CAPS buffer and electro-blotting was carried out at a constant current of 150 mA for 3 h at 4 ºC. For transferring large proteins (>150 kDa) electro-blotting was carried out at a constant current of 40 mA for 22.5 h at 4 ºC.

After electroblotting, the PVDF membrane was imaged again using the Chemi-Doc system to detect total protein content on the blot if a Mini-PROTEAN TGX stain free, AnyKD gradient gel (BIO-RAD) was used.

2.3.2.5 Blocking

The the PVDF membrane was blocked with 5 % skim milk in 1x TBS-T (20 mM Tris-Base, 137 mM NaCl and 0.1% Tween-20) at room temperature for 1 h with gentle rocking.

2.3.2.6 Incubating with antibodies

Primary antibodies were diluted appropriately in 1% skim milk in 1x TBS-T, added to the blot and incubated overnight at 4 ºC with gentle rocking. The blot was washed three times with 1xTBS-T the next day, and incubated with horseradish peroxidise conjugated secondary antibodies at room temperature for 1 h.

A variety of primary antibodies were tested at different concentrations until the best antibody and optimal concentration was determined. Some of these antibodies were validated using siRNAs. For list of antibodies used refer to table 2.5.

2.3.2.7 Detecting bands

The enhanced chemiluminescence (ECL) (GE health care) system was used to detect bands, which were imaged with a Chemi-Doc MP system (Bio-Rad). Densitometry was performed using the band analysis tools of ImageLab software version 4.0 (BioRad). The linearity of ECL signal was established by loading a serial dilution of total protein (10, 20, 30, and 40 μg) and measuring the ECL signal. The ECL signal of the serial dilution of total protein increased in a linear manner. Protein levels were normalised to β-tubulin, α-actinin or total protein levels. Total protein levels or β-tubulin or α-actinin levels did not change between different treatments. The normalisation was performed following previously published protocol by (Taylor et al., 2013) Quantified results represent n=3 samples and these were technical replicates. Statistical significance was determined using Student's t-test.

Table 2.6 List of antibodies

Antigen	Molecular weight	Clonality; clone; host species	Dilution	Source/ reference
DICER	217 kDa	Polyclonal, Rabbit	1:1000	Cell Signalling Technology
DROSHA	160 kDa	Monoclonal, Rabbit	1:1000	Cell Signalling Technology
Exportin-5	136 kDa	Monoclonal, Rabbit	1:1000	Abcam
TARBP2	55 kDa	Monoclonal, Mouse	1:1000	Abnova
AGO2	97 kDa	Monoclonal, Rabbit	1:500	anti-Ago2(11A9) (Rudel et al., 2008)
PHD1	45 kDa	Polyclonal, Rabbit	1:1000	Novus Biologicals
PHD2	46 kDa	Polyclonal, Rabbit	1:1000	Abcam
FIH-1	40 kDa	Monoclonal, Mouse clone FIH 162c	1:1000	Millipore
β-tubulin	55 kDa	Polyclonal, Mouse	1:20000	Sigma
α-actinin	100 kDa	Polyclonal, Rabbit	1:500	Cell Signalling Technology
Horseradish peroxidise conjugated secondary antibodies goat anti-rabbit IgG		Goat	1:5000	Immunopure: Thermo Scientific
Horseradish peroxidise conjugated secondary antibodies donkey anti-mouse IgG		Goat	1:5000	Immunopure: Thermo Scientific

2.4 Measurement of luciferase acitivity in transiently transfected cells

Luciferase activity was detected using a dual-luciferase reporter assay system (Promega). Cells transfected with plasmids containing a luciferase reporter were lysed by adding the supplied cell lysis buffer and incubated at room temperature for 15 min, before being mixed well by pipetting. Cell lysates were collected in an eppendorf tube and centrifuged at 10 000 rpm to collect cell debris. The cleared lysates were used to measure luciferase activity after adding 100 μL Luciferase Assay Reagent. Luminescence was measured using a plate reader luminometer (Beckman Coulter DTX 880 Multimode detector).

2.5 DICER activity assay

In vitro DICER activity assay was performed following the previously described method (Perron et al., 2011). A plasmid containing pre-miR-145 with a T7 RNA polymerase promoter was used to prepare the substrate RNA. The plasmid DNA was linearized with a restriction enzyme downstream of the insert to ensure transcription was terminated by run-off. A restriction enzyme for linearization one that leaves a 5' overhang was selected, A previous report showed low levels of transcription with plasmid template strands cut with restriction enzymes leaving 3' overhangs (Schendorn and Mierindorf, 1985). The linearized plasmid DNA was run on a 0.5% Agarose gel to confirm that cleavage was complete, because even a small amount of circular plasmid in the template would generate a large proportion of longer transcripts.

2.5.1 Gel purification of plasmid DNA

The linearized plasmid DNA band was cut out of the gel under UV light and purified using a Qiagen QIAquick gel extraction kit following manufacture's protocol.

2.5.2 *In Vitro* Transcription for RNA Radio-labelling with UTP

2.5.2.1 MEGAshortscript protocol

The reaction was assembled in RNase-free microfuge tube at room temperature (Components in the transcription buffer can lead to precipitation of the template DNA if the reaction is assembled on ice) in the order shown in the manufacture's protocol. The contents were mixed thoroughly by gently flicking the tube and then spun briefly to collect the reaction mixture at the bottom of the tube.

Per reaction: 2 µL T7 10X reaction buffer; 2 µL T7 ATP Solution (75 mM); 2 µL T7 CTP Solution (75 mM); 2 µL T7 GTP Solution (75 mM); 20 µCi of α ^{32}P; <8 µL Template DNA; 2 µL T7 enzyme mix and nuclease-free water up to 20 µL final volume. Reaction mix was incubated at 37°C for 3 h.

To remove excess DNA template, 1 µL of TURBO DNase was added to the reaction, mixed well and continued the incubation at 37°C for a further 15 min. This reaction was stopped by adding 1 µL of EDTA 0.5 M, mixed and incubated for 2 min at room temperature. 20 µL of gel loading buffer (GLB) II (provided in the MEGAshort script T7 kit) was added to the mix, heated for 5 min at 95°C and quickly put on ice for 5 min.

2.5.2.2 Resolving RNA on a denaturing PAGE gel

All of the reaction mix was loaded on to a denaturing PAGE gel. Electrophoresis was conducted at a constant voltage of 275 V for ~2 h or until the bromophenol blue was 2 cm from the bottom of gel. TBE (x1) was used as the gel running buffer. When the migration is finished, the gel was left on one of the two glass plates and wrapped using plastic wrap. The gel was placed in a plexiglass box. In the dark room, the gel was exposed to an X-ray film for 2 min and 10 min. Afterwards, in the radioactivity room, the band corresponding to the RNA probe was cut out.

2.5.2.3 Extracting RNA from the gel

The gel slice was cut in to small pieces and put into a 1.5-mL screwcap tube. 400 μL of gel extraction buffer was added and incubated overnight at 37°C. Next morning the tube was centrifuged at 600 × *g* for 1 min at 4°C. The supernatant was transferred into a new 1.5-mL screw-cap tube. 1 μL glycogen and 1 mL of ice-cold ethanol (100%) was added to the supernatant, mixed well and incubated overnight at −80°C. The next morning the tube was centrifuged at 16,000 × *g* for 30 min at 4°C. The supernatant was removed with a pipette and the pellet was washed with 900 μL of ice-cold ethanol (70%) and then centrifuged at 16,000 × *g* for 5 min at 4°C.

The RNA pellet (DICER substrate probe) was dried and resuspend in 20 μL of annealing buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 25 mM NaCl). The RNA probe was annealed by heating for 5 min at 85°C and then removed from the heating block and allowed to cool down to room temperature. 1 μL of the RNA solution was added to 5 mL of scintillation liquid and counted using a beta scintillation counter. ^{32}P UTP labelled sample was aliquoted into different tubes (to be used at 40,000 cpm/μL) and stored at −80°C.

2.5.3 End labelling Decade markers

The Ambion® Decade™ Marker System produces a set of radiolabeled RNA molecules of 150, 100, 90, 80, 70, 60, 50, 40, 30, 20, and 10 nucleotides. The system included all of the reagents necessary to produce the molecular weight markers except gamma-^{32}P-ATP. The Decade™ Markers derive from a single, gel-purified transcript that was provided in the kit. The transcript was 5' end-labelled using the kinase and kinase buffer supplied with the kit. The kinase reaction was then diluted

into a cleavage reagent that generated the molecular weight marker set in a five minute, room temperature reaction. Gel loading buffer from the kit was then added and the marker is ready for electrophoresis.

2.5.4 Detection of human DICER Activity

This protocol was adapted from Haase et al. (2009). Mammalian cells (MCF7) cultured in a 100-mm petri dish were lysed by adding 250 μL of DICER lysis buffer *(*20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, and 0.25% NP-40. 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease cocktail inhibitor without EDTA 1× (Roche) added just prior to use) and then incubated for 15 min on ice and centrifuged at 10,000 × *g* for 10 min at 4°C.

The protein concentration was determined by EZQ protein quantification assay method as described previously. The protein concentration was adjusted to 5 mg/mL with DICER lysis buffer.

A 50 μL reaction was prepared on ice in 1.5-mL screw-cap tubes as follows: 25 μL of MCF7 cell extract at 5 mg/mL (125 μg protein in total), 24 μL of *2*× DICER assay buffer *(*20 mM Tris–HCl pH 7.5, 2 mM MgCl2, 75 mM NaCl, 10% glycerol. Add 1 mM PMSF and protease inhibitor cocktail mix without EDTA 1×, prior to use), and 1 μL of radiolabeled dsRNA substrate probe (40,000 cpm/μL). The reaction was mixed by vortexing and then incubated for 10, 30, 60, and 120 min at 37°C.

To stop the reaction, 150 μL of water and 200 μL of Acid Phenol:CHCl3 (5:1 solution, pH 4.5) was added, vortexed for 20 s and the aqueous and organic phases were separated by centrifugation at 16,000 × *g* for 4 min at 4°C.

The aqueous phase was transferred to a fresh tube and RNA was precipitated by adding 50 µL of 5 M ammonium acetate, 700 µL of ice-cold ethanol (100%), 1 µL of yeast tRNA, and 1 µL of glycogen and then incubated overnight at −80°C. Next morning the reaction mix was centrifuged at 16,000 × g for 30 min at 4°C, the supernatant was removed and RNA pellet was washed with 400 µL of ice-cold ethanol (70%) before being centrifuged at 16,000 × g for 5 min at 4°C. The RNA pellet was dried at room temperature for 5 min, before 10 µL of GLB II was added, and heated for 5 min at 95°C. The whole sample was loaded on a denaturing PAGE gel. After electrophoresis the gel was wrapped in a plastic wrap and exposed to an x-ray film with an intensifying screen at −80°C.

The developed x-ray film was imaged with a Chemi-Doc MP system (Bio-Rad). Densitometry was performed using the ImageLab software version 4.0 (BioRad). Pre-miR-145 substrate RNA levels were normalised to the starting RNA concentrations.

2.5.5 Preparing a denaturing PAGE gel

Preparing a 0.75-mm thick 10% polyacrylamide gel (19:1) containing 7 M of urea. For a 30-mL preparation, 7.5 mL of polyacrylamide (19:1) (stock 40%), 12.6 g of urea, 3 mL of 10×TBE solution was mixed with nuclease free water. To completely dissolve urea the mix was incubated at 37°C. Once the urea was dissolved 150 µL of 10% APS and 30 µL of TEMED was added, mixed gently, and poured immediately. The gel polymerized in approximately 30 min.

The gel was pre-run at 250 V for 30 min in 1× TBE at room temperature. Electrophoresis apparatus with a big tank assisted with a constant migration

temperature. The wells were rinsed two times with 1× TBE buffer before loading the RNA samples. A radiolabeled Decade size marker was loaded in order to estimate the size of RNA species under study. Electrophoresis was conducted at a constant 275 V for 1.5 h or until the bromophenol blue was 2 cm from the bottom of the gel.

Chapter 3 . DICER regulation by hypoxia

3.1 Introduction

DICER is a key ribonuclease III enzyme involved in the maturation of miRNAs and the cleavage of double stranded RNAs into small interfering RNAs. In the cytoplasm precursor miRNAs are cleaved by DICER (Grishok et al., 2001, Hutvagner et al., 2001), coupled with TARBP2 protein (Chendrimada et al., 2005, Haase et al., 2005). DICER is also an integral part of the RISC loading complex, that assist in loading the mature miRNA strand on to the RISC, which is involved in post-translational gene regulation (Gregory et al., 2005, Hammond et al., 2000, Chendrimada et al., 2005). DICER cleavage is essential for processing most miRNAs and deletion of DICER decreases mature miRNA expression (Grishok et al., 2001, Hutvagner et al., 2001). A reduction in mature miRNA levels have been observed after inhibiting *DICER* using RNA interference or in DICER null cell lines (Ravi et al., 2012, Sadegh et al., 2012, Suarez et al., 2007).

There is some evidence from previous work suggesting that hypoxia may be involved in regulating DICER (Caruso et al., 2010, Elvidge et al., 2006). A microarray study looking at the mRNA expression in breast cancer cell line MCF7 after exposure to hypoxia showed a modest but consistent decrease in *DICER* mRNA levels (Elvidge et al., 2006). In addition, Caruso et al. (2010) reported a decrease in DICER expression during the development of pulmonary arterial hypertension in rats after exposure to chronic hypoxia (Caruso et al., 2010).

Considering the importance of DICER in miRNA biogenesis, and the possible links between hypoxia and DICER expression, a detailed examination of DICER mRNA and protein levels in hypoxia was conducted using a cancer cell model. As discussed

in chapter 1, gene regulation in hypoxia may be mediated through transcriptional (Wang and Semenza, 1995, Wang and Semenza, 1993, Wang et al., 1995) and post-transcriptional mechanisms (Liu et al., 2006, Goldberg et al., 1991). To investigate the effects of hypoxia on *DICER* transcription/mRNA stability, *DICER* mRNA levels were examined in three different cancer cell lines (MCF7, HT29 and SKBR3) after exposure to hypoxia. To identify possible effects of hypoxia on post-transcriptional, translational and protein degradation mechanisms, DICER protein levels were examined in cancer cell lines (MCF7 and SKBR3) and a non-cancer cell line (HUVECs) after exposure to hypoxia. In all *in vitro* experiments, cells were exposed to hypoxic conditions (0.1% or 1 % O_2) and compared with controls under normoxic conditions (21% O_2).

These experimental conditions were similar to O_2 tension in hypoxic tumours, and have been commonly used in cell culture experiments that have defined action of HIF-1 and its O_2 sensing control (Elvidge et al., 2006). Cancer cell lines have been widely used in research studies and have proven to be a useful model for studying biological mechanisms involved in cancer (Louzada et al., 2012, van Staveren et al., 2009, Burdall et al., 2003). The MCF7 cell line was previously used in a microarray study by Elvidge et al. (2006), which showed *DICER* mRNA down regulation in hypoxia, and in this study MCF7 cell line was used to allow comparison between studies. Data presented in this chapter show DICER mRNA and protein levels after exposure to hypoxia, when compared to normoxia.

3.2 Aims

The main aims of this chapter were to identify the effect of hypoxia on *DICER* mRNA expression and DICER protein levels in cancer and non-cancer cells using real time RT-PCR and immunoblotting techniques.

3.3 Exposing cells to hypoxia

MCF7 cells were incubated in a hypoxic incubator at 0.1% O_2 for 16 h and control plates were incubated at normoxic conditions. After 16 h cells were harvested by adding TRIzol reagent, and RNA was extracted following the TRIzol protocol (see chapter 2). After hypoxic exposure media was removed and cells were lysed under hypoxia. Hypoxically induced carbonic anhydrase IX (*CAIX*) mRNA (Wykoff et al., 2000) levels and miRNA-210 (Camps et al., 2008) levels were quantified with real time RT-PCR. There was a large induction of *CAIX* mRNA levels (100-fold) (Figure 3.1) and miR-210 levels (2.6-fold) (Figure 3.2) in MCF7 cells exposed to hypoxia when compared to normoxia. Ribosomal RNA 18S was used to normalise mRNA expression and small nucleolar RNA, RNU6B, was used to normalise miRNA expression. These controls did not change in hypoxia compared to normoxia. The following initial results confirmed the induction of well characterised hypoxically regulated genes in this cell culture model of hypoxic exposure.

It is possible that the polystyrene plasticware used for cell culture contained some dissolved O_2. This might have slightly altered the level of O_2 the cells were exposed to, specifically the cells in hypoxic conditions. However, large induction of the hypoxically regulated mRNA *CAIX* and miR-210 levels in hypoxia confirms the

hypoxic exposure of these cells. These observations suggest that the dissolved O_2 in plasticware did not significantly affect the gene expression of cells in hypoxia when compared to normoxia.

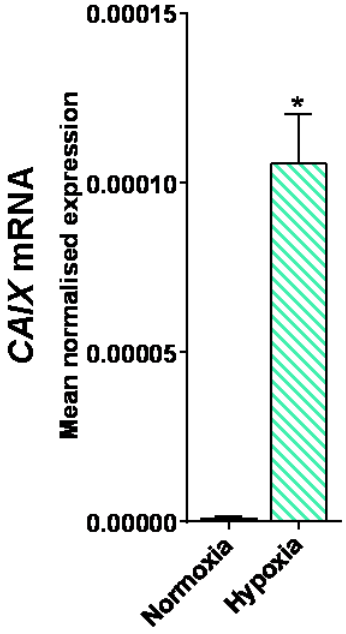

Figure 3.1 *CAIX* mRNA expression in MCF7 cells in hypoxia vs. normoxia.

CAIX mRNA levels were analysed by real time RT-PCR and normalised to 18S ribosomal RNA levels. *CAIX* mRNA expression in MCF7 cells after hypoxia (0.1% O_2 for 16 h) vs. normoxia (P=0.0001). Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $p < 0.05$ compared with parallel controls.

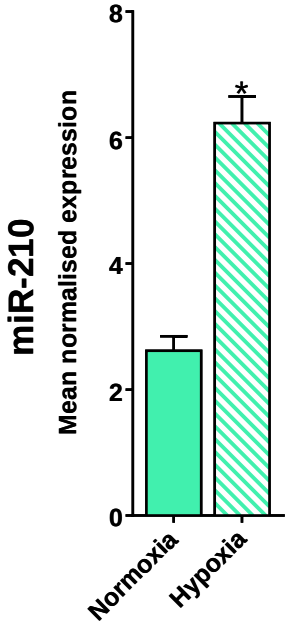

Figure 3.2 miR-210 expression in MCF7 cells in hypoxia vs. normoxia.

miR-210 levels were analysed by real time RT-PCR and normalised to RNU6B small nucleolar RNA levels. miR-210 expression in MCF7 cells after hypoxia (0.1% O_2 for 16 h) vs. normoxia (P=0.0002). Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $p < 0.05$ compared with parallel controls.

3.4 Hypoxic regulation of *DICER* mRNA expression

3.4.1 The effect of hypoxia on *DICER* mRNA expression in cancer cell lines

3.4.1.1 Exposing cells to different concentrations of hypoxia

Initially, MCF7 cells were exposed to different levels of hypoxia (0.1%, 1%, 2% and 5% O_2) for 16 h with parallel controls in normoxia (21% O_2). The aim of this initial experiment was to identify the O_2 concentration that had a significant effect on *DICER* mRNA expression. Cells were harvested after the 16 h incubation, and RNA was extracted following the TRIzol method (See chapter 2). Relative quantitation real time RT-PCR was used to determine *DICER* mRNA expression. The ribosomal RNA 18S was used as the normalising control and 18S levels did not change in hypoxia, compared to normoxia. The most significant reduction of *DICER* mRNA expression was seen at 0.1% O_2 after 16 h (P=0.0009) (Figure 3.3). No significant changes in *DICER* mRNA levels were observed at O_2 concentrations of 1%, 2% or 5% after 16 h (Figure 3.3).

To confirm that non-specific stress and toxic effects due to hypoxia did not affect the cell viability, a cell viability assay was done comparing hypoxic and normoxic treatments. The cell viability assay showed that there were no significant changes in the cell viability of SKBR3 cells between hypoxic (1% O_2 for 48 h) and normoxic cultures (Figure 3.4). Cell viability assays done at much lower O_2 concentrations such as 0.1% O_2 for 48 h did not show a significant change in cell viability in SKBR3, MCF7 or HT29 cell lines (work conducted by other members of the lab). In addition there was no change in any of the controls (miRNA, mRNA and protein levels) between hypoxia and normoxia, further confirming the lack of toxic effects due to non-specific strees in cells during the course of these experiments.

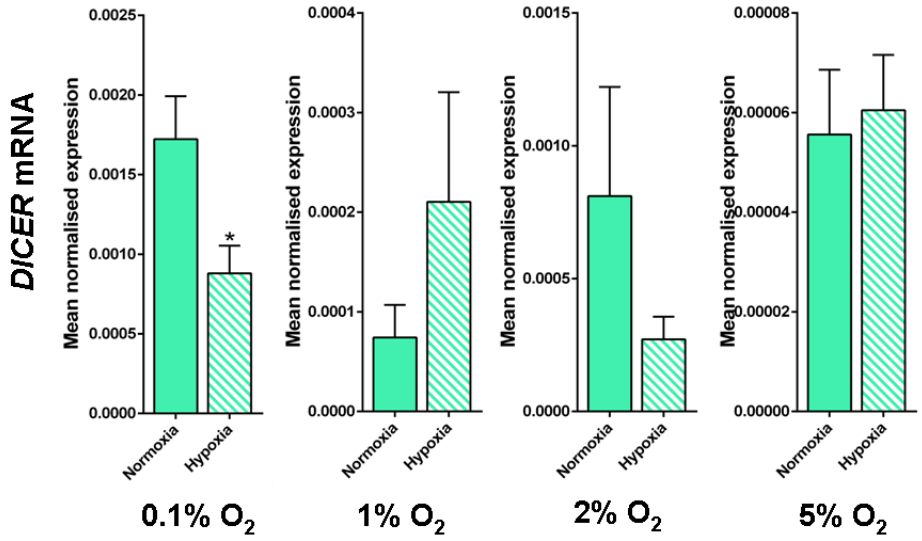

Figure 3.3 *DICER* mRNA expression in MCF7 cells after exposure to varying concentrations of hypoxia.

DICER mRNA levels were analysed by real time RT-PCR and normalised to 18S ribosomal RNA levels. *DICER* mRNA expression in MCF7 cells after exposure to 16 h for 0.1% O_2 (P=0.0009), 1% O_2, 2% O_2 and 5% O_2. Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance examined for with student's t-test. * denotes P < 0.05 compared with parallel controls in normoxia.

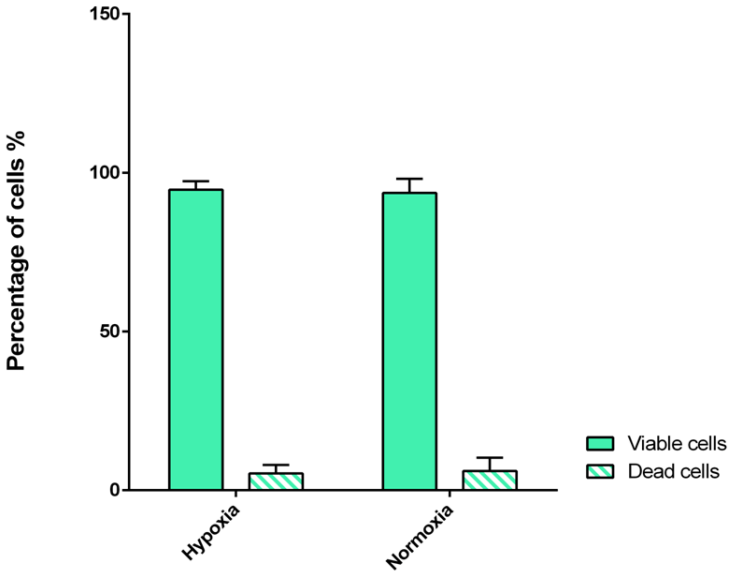

Figure 3.4 Viability of SKBR3 cells following hypoxia.

Viability of SKBR3 cells was measured using the trypan blue exclusion method. Cells were exposed to hypoxia (1% O_2 for 48 h) vs. normoxia, and viability was determined using the trypan blue exclusion method. Data represent mean ±S.E (error bars) (n=6).

3.4.1.2 Exposing cells to varying durations of hypoxia

After observing a significant decrease in *DICER* mRNA expression at 0.1% O_2 after 16 h, MCF7 cells were exposed to varying durations of hypoxia vs. normoxia, to examine the time course of hypoxic down regulation of *DICER* mRNA expression. MCF7 cells were exposed to 8 h, 16 h, 24 h and 48 h of hypoxia (0.1% O_2), compared to normoxia. *DICER* mRNA expression over the time course was quantified in hypoxia compared to normoxia using real time RT-PCR. There was a significant and consistent repression of *DICER* mRNA levels after exposure to hypoxia (0.1% O_2) for 8 h (P=0.007), 16 h (P=0.0009), 24 h (P=0.02) and 48 h (P=0.008) in MCF7 cells (Figure 3.5), when compared to normoxia.

To investigate if this repression was observed in other cancer cell lines, DICER mRNA levels were also examined in the colorectal cancer cell line HT29. HT29 cells were exposed to hypoxia (0.1% O_2), compared to normoxia for 8 h, 16 h, 24 h and 48 h and *DICER* mRNA expression was quantified using real time RT-PCR. A similar, albeit less striking, reduction in *DICER* mRNA levels was seen in HT29 cells after exposure to hypoxia (0.1% O_2) for 8 h, (P=0.2), 16 h (P=0.01), 24 h (P=0.02) and 48 h (P=0.09) when compared to normoxia (Figure 3.6).

No significant changes in *DICER* mRNA levels were observed in SKBR3 cells after being exposed to 0.1% O_2 for 24 h and 36 h or after being exposed to 1% O_2 for 72 h (Figure 3.7).

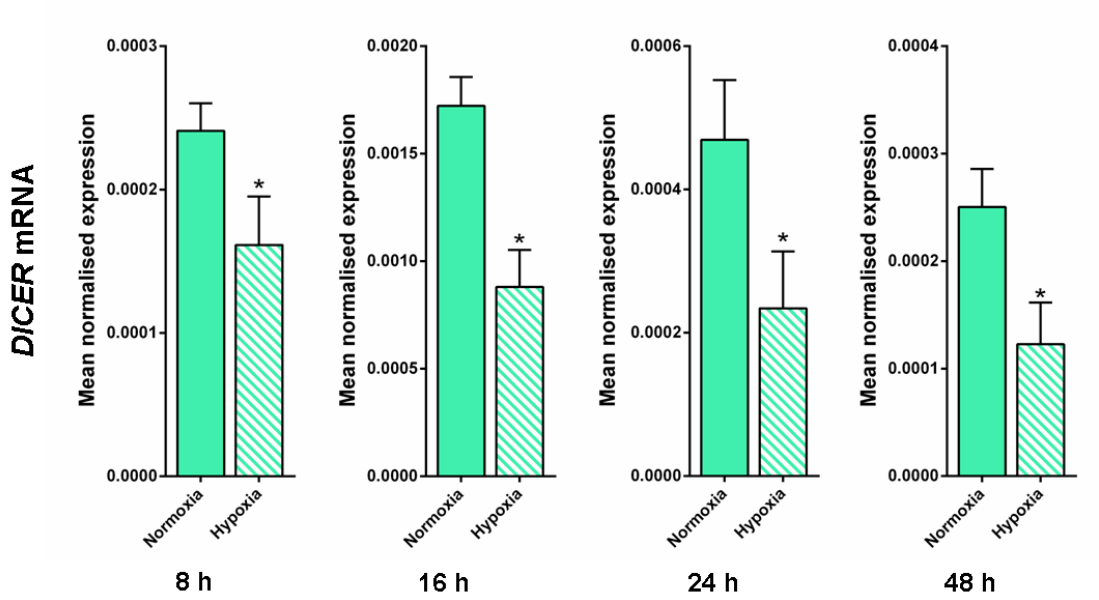

Figure 3.5 *DICER* mRNA expression in MCF7 cells in hypoxia vs. normoxia.

DICER mRNA levels were analysed by real time RT-PCR and normalised to 18S ribosomal RNA levels. *DICER* mRNA expression in MCF7 cells after exposure to 0.1% O_2 for 8 h (P=0.007), 16 h (P=0.0009), 24 h (P=0.02) and 48 h (P=0.008). Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes P < 0.05 compared with parallel controls in normoxia.

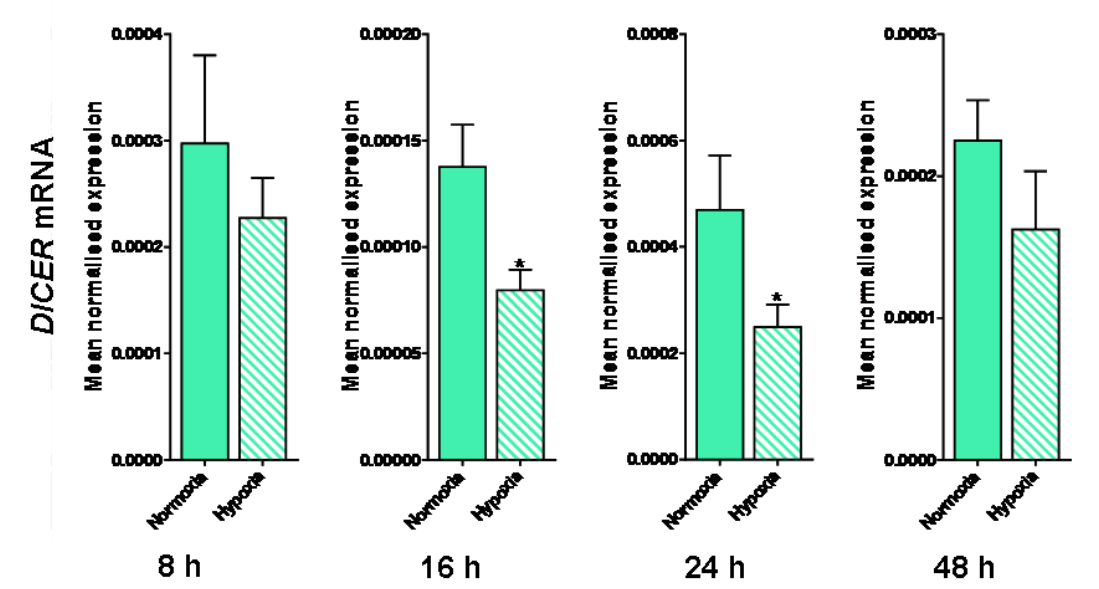

Figure 3.6 *DICER* mRNA expression in HT29 cells in hypoxia vs. normoxia.

DICER mRNA levels were analysed by real time RT-PCR and normalised to 18S ribosomal RNA levels. *DICER* mRNA expression in HT29 cells after exposure to 0.1% O_2 for 8 h (P=0.2), 16 h (P=0.01), 24 h (P=0.02) and 48 h (P=0.09). Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes P < 0.05 compared with parallel controls in normoxia.

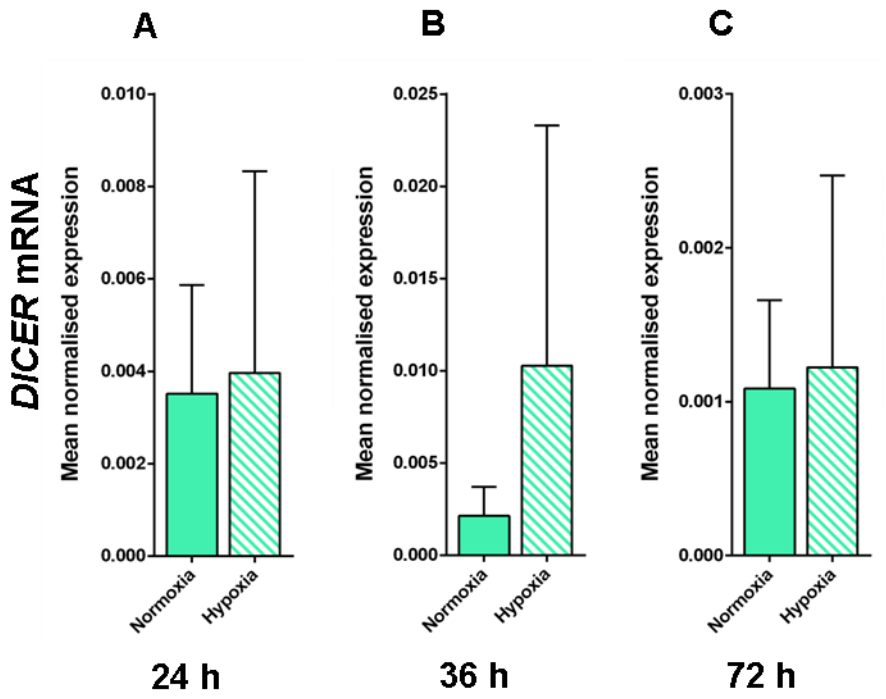

Figure 3.7 *DICER* mRNA expression in SKBR3 cells in hypoxia vs. normoxia.

DICER mRNA levels were analysed by real time RT-PCR and normalised to 18S ribosomal RNA levels. A, *DICER* mRNA expression in SKBR3 cells after exposure to 0.1% O_2 for 24 h and B, 36 h. C, *DICER* mRNA expression in SKBR3 cells after exposure to 1% O_2 for 72 h. Data represent normalized mean ±S.E (error bars) (n=3).

3.5 Hypoxic regulation of DICER protein levels

3.5.1 The effect of hypoxia on DICER protein levels in breast cancer cell lines

To examine whether the hypoxic repression of *DICER* mRNA levels was reflected in alterations in DICER protein levels, two different breast cancer cell lines (MCF7 and SKBR3 cells) were exposed to varying durations (16. 24 and 48 h) of hypoxia (0.1% O_2). The changes in protein levels were analysed by immunoblotting. α-actinin protein level was used as a loading control, and hypoxia did not affect α-actinin level in the different cell lines examined in this study. After 16 h and 24 h of hypoxia no or very modest reductions in DICER protein abundance were seen in SKBR3 cells (Figure 3.8 A and B) and MCF7 cells (Figure 3.8 C and D).

To accommodate the possibility that hypoxic alterations in *DICER* mRNA levels were not reflected by alterations in protein levels because of significant stability of the DICER protein, the effects of longer durations of hypoxia on MCF7 and SKBR3 cells were examined. A striking reduction in DICER protein levels was seen in both MCF7 (5-fold) (Figure 3.9A) and SKBR3 cells (40-fold) (Figure 3.9B) after being exposed to 0.1% O_2 for 48 h. The expression of the control protein α-actinin was unaffected by the hypoxic exposure. A large reduction of DICER protein was also observed in MCF7 cells (4.5-fold) (Figure 3.10A) and SKBR3 cells (5.4-fold) (Figure 3.10B) even after a more modest hypoxic exposure (1% O_2) for 72 h.

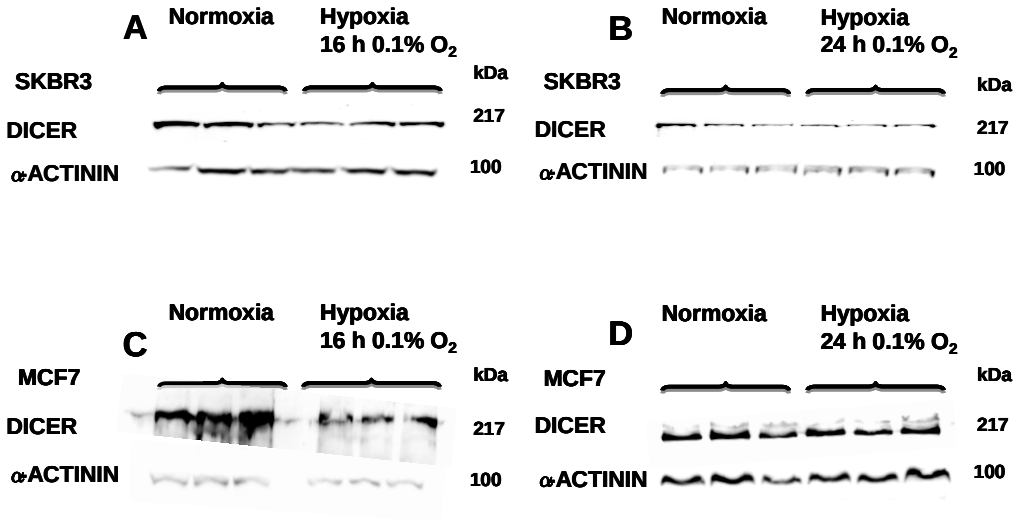

Figure 3.8 DICER protein levels in MCF7 and SKBR3 cells in hypoxia vs. normoxia.

DICER and α-actinin protein level was examined by immunoblotting. A, DICER protein levels in SKBR3 cells after exposure to 0.1% O_2 for 16 h and B, 24 h. C, DICER protein levels in MCF7 cells after exposure to 0.1% O_2 for 16 h, D, 24 h.

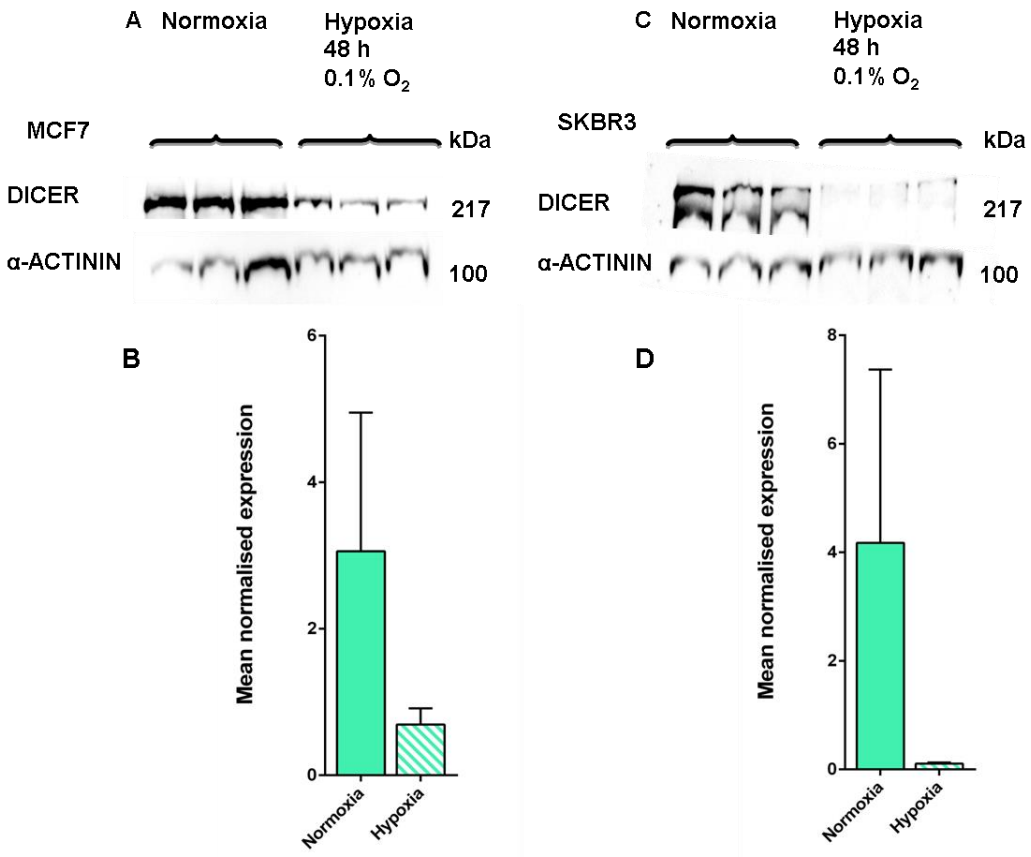

Figure 3.9 DICER protein levels in MCF7 and SKBR3 cells hypoxia vs. normoxia.

DICER and α-actinin protein levels were examined by immunoblotting. A, DICER protein levels in MCF7 cells after exposure to 0.1% O_2 for 48 h. B, Densitometric analysis of A following normalisation with α-actinin levels. C, DICER protein levels in SKBR3 cells after exposure to 0.1% O_2 for 48 h. D, Densitometric analysis of B following normalisation with α-actinin levels. Data represent normalized mean ±S.E (error bars) (n=3).

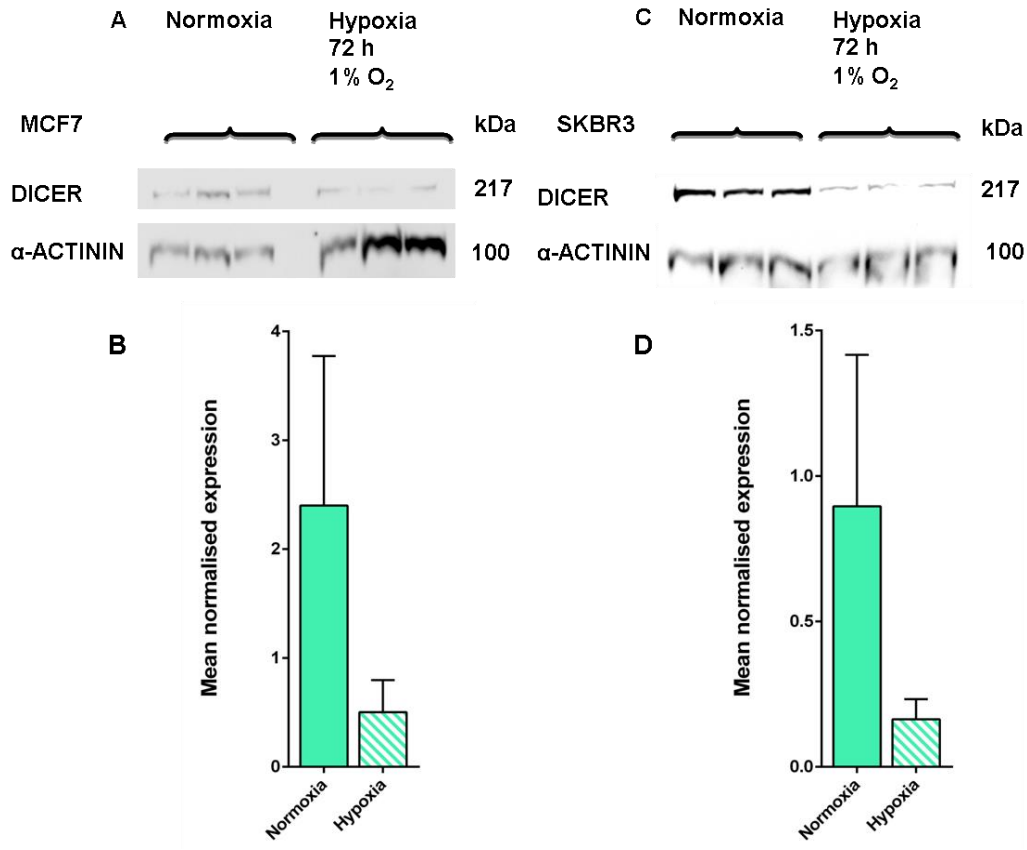

Figure 3.10 DICER protein levels in MCF7 and SKBR3 cells hypoxia vs. normoxia.

DICER and α-actinin protein levels were examined by immunoblotting. A, DICER protein levels in MCF7 cells after exposure to 1% O_2 for 72 h. B, Densitometric analysis of A following normalisation with α-actinin levels. **C**, DICER protein levels in SKBR3 cells after exposure to 1% O_2 for 72 h. D, Densitometric analysis of B following normalisation with α-actinin levels. Data represent normalized mean ±S.E (error bars) (n=3).

3.5.2 The effect of hypoxia on DICER protein levels in human umbilical vein endothelial cells (HUVECs)

To examine whether hypoxic repression of DICER was also observed in non-cancer cells, human umbilical vein endothelial cells (HUVECs) were studied. HUVECs are used in most research directed towards the development of anti-angiogenic and anti-cancer agents (Lai et al., 2012). HUVECs from multiple donors were exposed to hypoxia (1% O_2) for different durations (24 h and 48 h). There was no change in DICER protein levels in HUVECs after 1% O_2 for 24 h (Figure 3.11A). A larger decrease (7-fold) in DICER protein levels was seen after 1% O_2 for 48 h (Figure 3.11B).

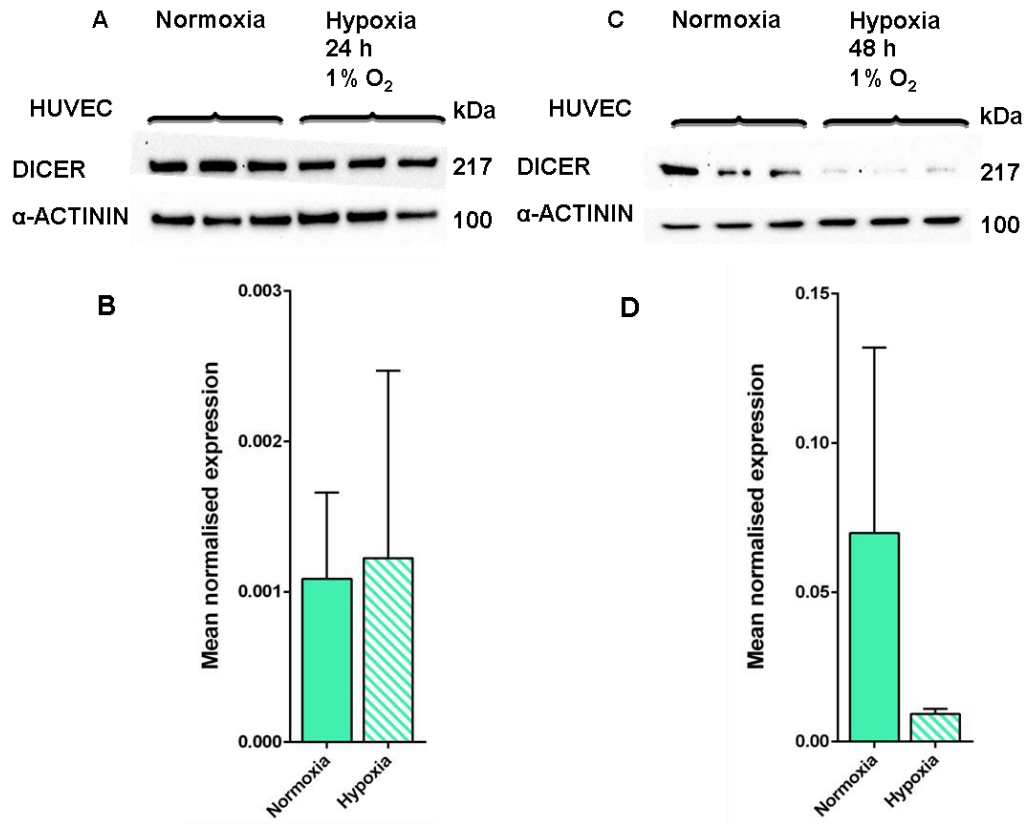

Figure 3.11 DICER protein levels in HUVECs in hypoxia vs. normoxia.

DICER and α-actinin protein levels were examined by immunoblotting. A, DICER protein levels in HUVECs after exposure to 1% O_2 for 24 h. B, Densitometric analysis of A following normalisation with α-actinin levels. C, DICER protein levels in HUVECs after exposure to 1% O_2 for 48 h. D, Densitometric analysis of B following normalisation with α-actinin levels. Data represent normalized mean ±S.E (error bars) (n=3).

3.6 Discussion

This chapter demonstrated the hypoxic down regulation of DICER, a key miRNA biogenesis protein. Hypoxia down regulated *DICER* mRNA levels in two different cancer cell lines, a breast cancer cell line (MCF7) and a colorectal cancer cell line (HT29). Significant down regulation of *DICER* mRNA levels were observed in MCF7 cells after exposure to hypoxia (0.1% O_2) for varying durations. Despite a downward trend in *DICER* mRNA levels in HT29 cells after exposure to hypoxia (0.1% O_2), these changes were less significant, when compared to results from MCF7 cells. In SKBR3 cells there were no significant changes in *DICER* mRNA levels after exposure to hypoxia. The hypoxic suppression of Dicer mRNA levels appears to account for regulation in some (MCF7 and HT29) but not all cells (e.g. SKBR3) indicating the operation of other mechanisms in the hypoxic regulation of Dicer.

Significant reductions in DICER protein levels were observed in MCF7 and SKBR3 cells only following longer durations of hypoxia (0.1% O_2 for 48 h or 1% O_2 for 72 h) Similarly, a reduction in DICER protein levels was observed only after 48 h of hypoxia (1% O_2) in the non-cancer HUVECs. The reductions in Dicer protein levels were much more significant following longer durations of hypoxia (for 48 and 72 h) and with greater severity of hypoxic exposure (0.1% O_2). One likely explanation for this observation is high stability of Dicer protein. Therefore, the decrease in transcription might not be readily evident until 48 h. Consistent with this explanation, only modest reductions in Dicer protein levels were observed at shorter durations (16 and 24 h) of hypoxic exposure.

Results from this study are in agreement with previous work showing DICER mRNA and protein down regulation in hypoxia. Elvidge et al. (2006) reported *DICER* mRNA down regulation in MCF7 cells after exposure to hypoxia. During the course of this work, hypoxic repression of DICER levels was reported by others (Caruso et al., 2010, Ho et al., 2012, Wu et al., 2011). A study using rat pulmonary artery fibroblasts exposed to chronic hypoxia showed reduced *DICER* mRNA expression (Caruso et al., 2010). Also in human pulmonary artery smooth muscle cells (PASMCs) DICER protein levels decreased after exposure to hypoxia (Wu et al., 2011). Ho et al. (2012) demonstrated DICER mRNA and protein down regulation in HUVECs after exposure to hypoxia (1% O_2) for 24 h.

In this thesis a reduction in DICER protein levels was only observed after exposure to hypoxia (1% O_2) for 48 h in HUVECs, and this observation was not in line with the previous observations by Ho et al. (2012). Differeing results may be due to the slight differences in the experimental conditions when exposing cells to hypoxia. In this study HUVECs were exposed to hypoxia using an incubator (see details in chapter 2) where precise O_2 levels could be controlled by balancing with CO_2, N_2 and room air. The use of an incubator with precisely controlled O_2 levels ensured all hypoxic exposures were consistent across different experiments allowing accurate comparisons between multiple experiments. Whereas Ho et al. (2012) used an anaerobic system in which O_2 levels were kept below 1% by flushing with an anaerobic gas mixture which did not allow for precise control.

Overall DICER levels were suppressed in cells exposed to hypoxia. This was observed in three different cancer cell lines, two breast cancer cell lines (SKBR3, MCF7) and one colorectal cancer cell line (HT29), suggesting that this could be a

common phenomenon in cancer cells. Furthermore, DICER down regulation was also observed in a non-cancer cell line (HUVECs), suggesting that this could be a common phenomenon in cells and not restricted to cancer cells. Whilst mRNA regulation by hypoxia was seen in MCF7 and HT29 cell lines it was not observed in SKBR3 cells, which displayed substantial hypoxic repression of DICER protein levels. This observation indicates the operation of different mechanisms of DICER regulation under hypoxia and this could involve transcriptional and/or post-transcriptional mechanisms.

3.7 Chapter summary

This chapter reports the effects of hypoxia on *DICER* mRNA and DICER protein levels in cancer and non-cancer cells, using real time RT-PCR and immunoblotting techniques. *DICER* mRNA levels were significantly down regulated in breast cancer cell line (MCF7) and colorectal cancer cell line (HT29) after exposure to hypoxia (0.1% O_2) for varying durations (8, 16, 24 and 48 h). Striking decreases in DICER protein levels were observed in breast cancer cell lines MCF7 and SKBR3 only after exposure to longer durations of hypoxia (0.1% O_2 for 48 h or 1% O_2 for 72 h). DICER protein down regulation was also observed in HUVECs, a non-cancer cell line after exposure to hypoxia.

Chapter 4 Mechanisms of DICER regulation by hypoxia

4.1 Introduction

Chapter 3 reported that there was a consistent down regulation of *DICER* mRNA levels in two cancer cell lines; breast cancer cell line MCF7 and colorectal cancer cell line HT29. Similarly down regulation of DICER protein levels was observed in two breast cancer cell lines (MCF7 and SKBR3) and a non-cancer human umbilical vein endothelial cell line (HUVEC). This chapter investigates the possible mechanisms involved in DICER down regulation in hypoxia by exploring the mechanisms involved in hypoxic regulation of transcription, translation and protein degradation.

As discussed in chapter 1, transcriptional gene regulation in hypoxia is mainly mediated by the transcription factor hypoxia inducible factor-1 (HIF-1) (Wang and Semenza, 1995, Wang and Semenza, 1993, Wang et al., 1995). HIF-1 may directly regulate genes at the transcriptional level by binding to hypoxia response elements in specific genes (Mole et al., 2009, Schodel et al., 2011, Semenza, 1998), or indirectly at the post-transcriptional or translational levels (Kulshreshtha et al., 2007a, Kulshreshtha et al., 2007b, Kaidi et al., 2007, Gustafsson et al., 2005, Koshiji et al., 2004, Uniacke et al., 2012). Even though HIF-1 is the main transcriptional regulator in hypoxia, HIF-1 independent mechanisms might also be involved in DICER regulation during hypoxia. Post-transcriptional gene regulation in hypoxia is mediated through influencing mRNA stability and protein translation (Liu et al., 2006, Goldberg et al., 1991). Key mechanisms in regulating protein synthesis in hypoxia involve the phosphorylation of translation initiation factor eIF2α (eukaryotic initiation factor 2α), the inhibition of the elongation factor eEF2 (eukaryotic elongation factor 2) and suppression of mTOR (mammalian target of rapamycin).

The mTOR suppression results in suppression of its targets 4EBP1, $p70^{s6k}$ and rpS6, which result in decreased mRNA translation, thus decreased protein synthesis (Koumenis et al., 2002, Liu et al., 2006, Romero-Ruiz et al., 2012, Wouters and Koritzinsky, 2008).

Several studies have shown that some miRNAs are induced or repressed in tumour hypoxia and these are referred to as hypoxically regulated miRNAs (HRMs) (Kulshreshtha et al., 2007a, Kulshreshtha et al., 2007b). Hypoxically induced miR-210 has been detected in many tumours with poor prognosis and metastatic potential (Foekens et al., 2008, Camps et al., 2008). Previous work showed miRNAs were involved in DICER regulation through feedback loop mechanisms, whereby DICER dependent miRNAs (miR-103/107 and let-7a) were involved in regulating DICER levels (Martello et al., 2010, Forman et al., 2008). Forman et al. (2008) reported conserved miRNA target sites in the 3'UTR of DICER mRNA and showed that DICER was targeted by let-7a (Forman et al., 2008).

HIF-1 is involved in regulating a large number of genes in hypoxia (Manalo et al., 2005, Elvidge et al., 2006, Hu et al., 2003, Choi et al., 2008). This study examined for the role of HIF-1 in DICER down regulation in hypoxia. In one of the breast cancer cell lines (SKBR3) examined in this study, there was a lack of mRNA regulation by hypoxia even though there was a significant decrease in DICER protein levels (see chapter 3). This observation lead this study to investigate the post-transcriptional mechanisms involved in DICER regulation in hypoxia. Recent research showed PHD2 dependent but HIF independent down regulation of proteins in hypoxia via a PHD2 mediated phosphorylation and suppression of the eukaryotic elongation factor-2 (eEF2) activity and protein translation under acute hypoxia

(Romero-Ruiz et al., 2012). The possible involvement of HIF independent but prolyl hydroxylase dependent mechanisms in DICER regulation was investigated. As previous evidence suggests DICER regulation in hypoxia could also be mediated through miRNAs (Forman et al., 2008, Martello et al., 2010). Therefore the possible involvement of hypoxically regulated miRNAs in DICER down regulation in hypoxia was examined. As significant consistent down regulation of DICER protein levels was observed in all the different cell lines examined in this study, possible mechanisms involved in protein degradation were also investigated. The majority of mammalian proteins are hydrolysed by the proteasome so the involvement of proteasomes in DICER protein regulation in hypoxia was examined.

4.2 Aims

The aims of this chapter were to identify mechanisms of DICER mRNA and protein regulation in hypoxia.

4.3 Hypoxia inducible factor dependent regulation of DICER

4.3.1 DICER mRNA and protein levels after HIF hydroxylase inhibition

To examine for the role of the HIF pathway in the hypoxic repression of DICER, cells were exposed to the HIF hydroxylase inhibitors, dimethyloxalylglycine (DMOG) and desferrioxamine (DFO), which induce HIF-1α levels under normoxic conditions. DMOG is a competitive inhibitor of prolyl hydroxylases (PHDs) and stabilizes HIF-1α expression at normal oxygen tensions. Desferrioxamine stabilises HIF-1α by inhibition of prolyl hydroxylases through chelation of the Fe2+ bound to

the active site of PHD, which is required for enzyme activity. To confirm HIF induction after exposure to DMOG, HIF-1α protein levels in cells were detected by immunoblotting. There was an induction of HIF protein levels under normoxic conditions in SKBR3 cells after exposure to 1 mM DMOG for 16 h when compared to the control (Fig 4.1).

Consistent with a role for HIF hydroxylases in the repression of DICER mRNA (Elvidge et al., 2006), a modest decrease in DICER mRNA levels was seen after exposing MCF7 cells to DMOG (1 mM) for 48 h (Figure 4.2A) and desferrioxamine (0.1 mM) for 48 h (Figure 4.2B). However there was no change in DICER mRNA levels in SKBR3 cells exposed to DMOG (Figure 4.3A) or DFO (Figure 4.3B). This observation was consistent with previous results that DICER mRNA levels did not change after exposure to hypoxia vs. normoxia in SKBR3 cells (see chapter 3).

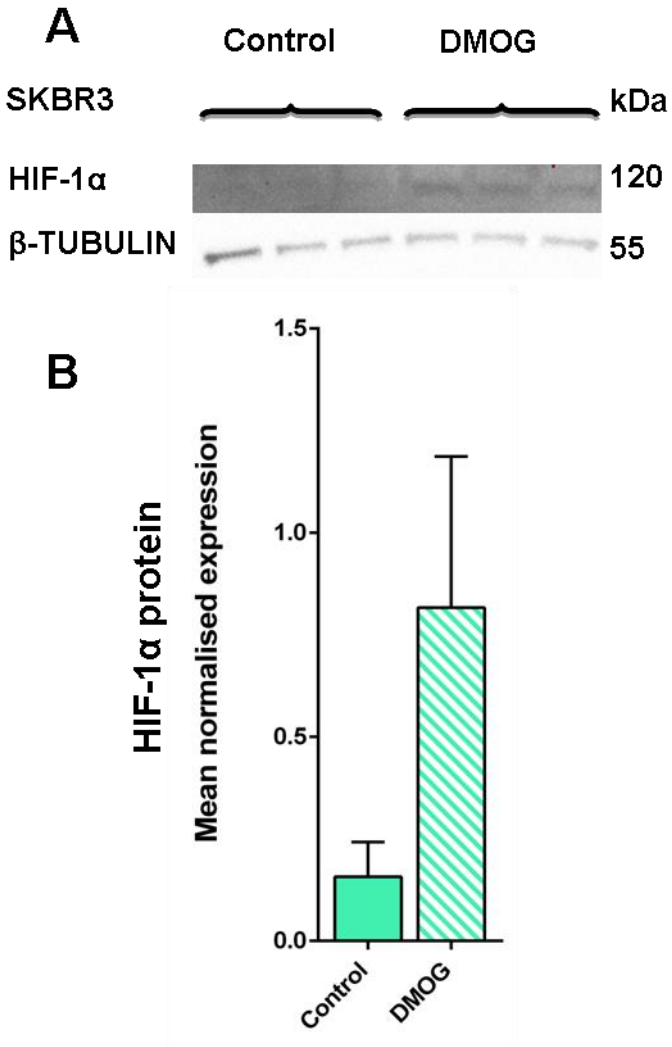

Figure 4.1 HIF-1α protein levels after exposure to HIF hydroxylase inhibitor dimethyloxalyl glycine (DMOG).

HIF-1α and β-tubulin protein levels were examined by immunoblotting. A, HIF-1α protein levels in SKBR3 cells after exposure to 1 mM DMOG for 16 h, compared with cells in control medium. β-tubulin was used as the loading control. B, Densitometric analysis following normalisation with β-tubulin levels. Data represent normalized mean ±S.E (error bars) (n=3).

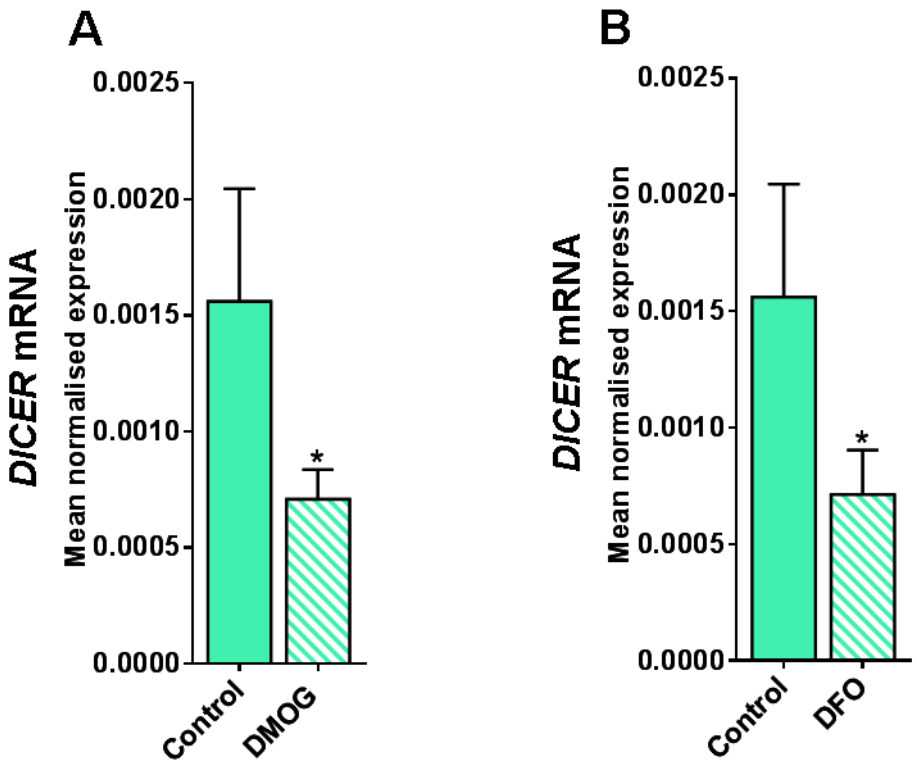

Figure 4.2 *DICER* mRNA expression in MCF7 cells after exposure to HIF hydroxylase inhibitors.

DICER mRNA levels were analysed by real time RT-PCR and normalised to 18S ribosomal RNA levels. A, *DICER* mRNA expression in MCF7 cells after exposure to 1 mM DMOG for 48 h, compared with cells in control medium (P=0.04). B, *DICER* mRNA expression in MCF7 cells after exposure to 0.1 mM DFO for 48 h, compared with cells in control medium (P=0.03). Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $P < 0.05$ compared with parallel controls.

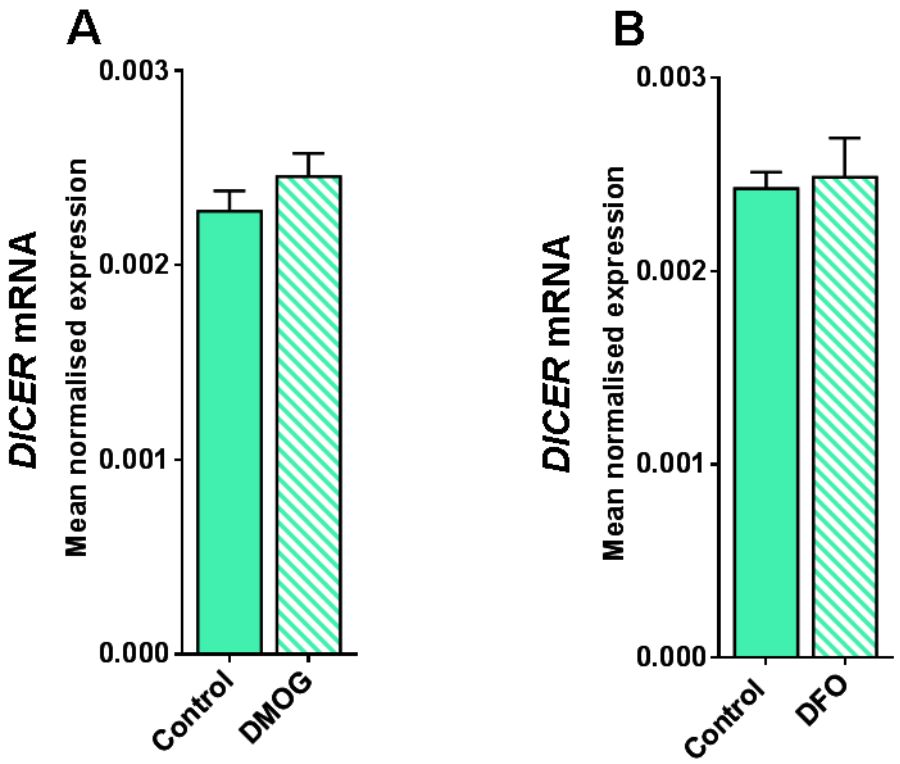

Figure 4.3 *DICER* mRNA expression in SKBR3 cells after exposure to HIF hydroxylase inhibitors.

DICER mRNA levels were analysed by real time RT-PCR and normalised to 18S ribosomal RNA levels. A, *DICER* mRNA expression in SKBR3 cells after exposure to 1 mM DMOG for 48 h. B, *DICER* mRNA expression in SKBR3 cells after exposure to 0.1 mM DFO for 48 h. Data represent normalized mean ±S.E (error bars) (n=3).

A similar effect on DICER protein levels was seen in cells following exposure to HIF hydroxylase inhibitors DMOG and desferrioxamine. A modest decrease in DICER protein levels was seen in MCF7 cells after exposure to DMOG (1 mM) (Figure 4.4A) for 48 h. Even though there was no change in DICER mRNA levels in SKBR3 cells after exposure to HIF hydroxylase inhibitor desferrioxamine (0.1 mM), a significant decrease in DICER protein levels was observed after 48 h (Figure 4.4B) This decrease suggests the involvement of post-transcriptional mechanisms in DICER regulation in SKBR3 cells in hypoxia.

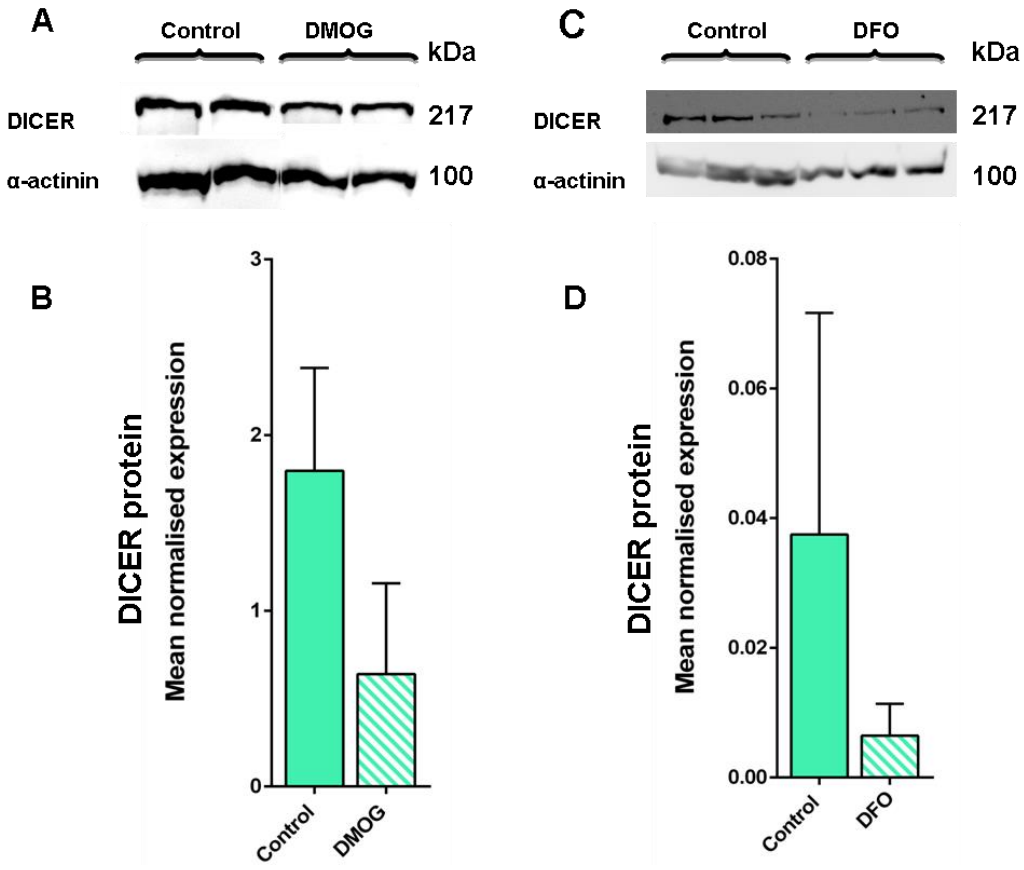

Figure 4.4 DICER protein levels in SKBR3 cells after exposure to HIF hydroxylase inhibitors.

DICER and α-actinin protein levels were examined by immunoblotting. A, DICER protein levels in SKBR3 cells after exposure to 1 mM DMOG for 48 h. B, Densitometric analysis of A following normalisation with α-actinin levels. C, DICER protein levels in SKBR3 cells after exposure to DFO (0.1 mM) for 48 h. D, Densitometric analysis of B following normalisation with α-actinin levels. Data represent normalized mean ±S.E (error bars) (n=3).

4.3.2 DICER mRNA and protein levels after HIF inhibition

To further investigate the involvement of HIF in DICER regulation the two main isoforms HIF-1α and HIF-2α were inhibited by siRNAs, and DICER expression in hypoxia compared to normoxia was examined. Cells were transiently transfected with siRNAs targeting HIF-1α, HIF-2α or control siRNAs and then exposed to hypoxia (0.1% O_2 for 48 h) vs. normoxia. There was substantial reduction in the hypoxic expression of HIF-1α protein in SKBR3 cells after transient transfection with siRNA targeting HIF-1α compared to the control siRNA (Figure 4.5).

After confirming the siRNA mediated suppression of HIF-1α expression in hypoxia, *DICER* mRNA expression was quantified by real time RT-PCR. HIF-1α inhibition had no effects on the repression of *DICER* mRNA in hypoxia in SKBR3 cells (Figure 4.6). Similarly, HIF-1α inhibition using siRNAs had no effect on the repression of *DICER* mRNA levels in hypoxia in MCF7 cells. HIF-2α expression is low in MCF7 cells so mainly the effect of HIF-1α was examined (Carroll and Ashcroft, 2006). Even when HIF-1α and HIF-2α were inhibited simultaneously using siRNAs there was no effect on the *DICER* mRNA repression in MCF7 cells (Figure 4.7), suggesting lack of HIF mediated down regulation of DICER mRNA levels in SKBR3 and MCF7 cell lines after exposure to hypoxia.

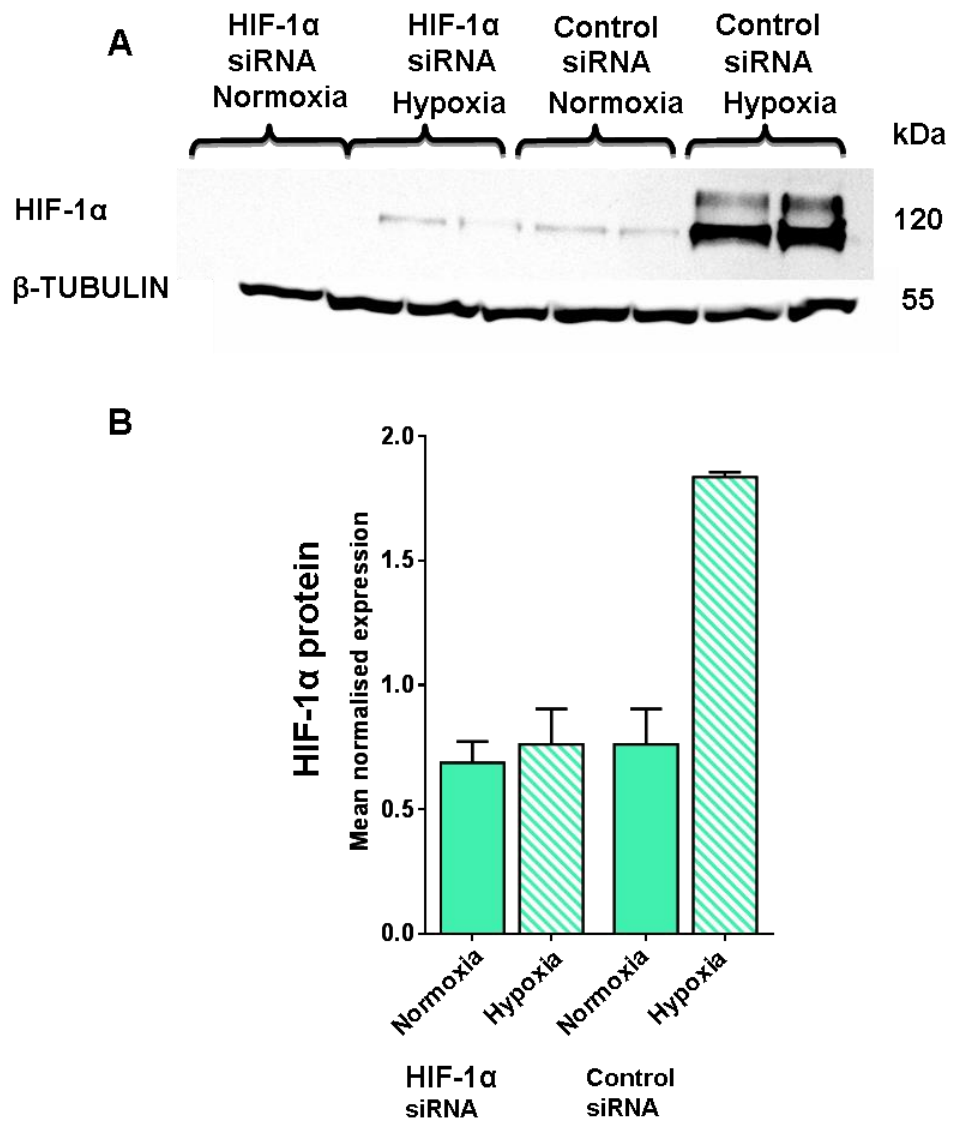

Figure 4.5 HIF-1 repression in SKBR3 cells after HIF-1α inhibition in hypoxia vs. normoxia.

HIF-1α and β-tubulin protein levels were examined by immunoblotting. A, HIF-1α protein levels in SKBR3 cells after transient transfection with a HIF-1α targeting siRNA or control siRNA, then exposure to hypoxia (0.1% O_2 for 24 h) vs. normoxia. B, Densitometric analysis following normalisation with β-tubulin.

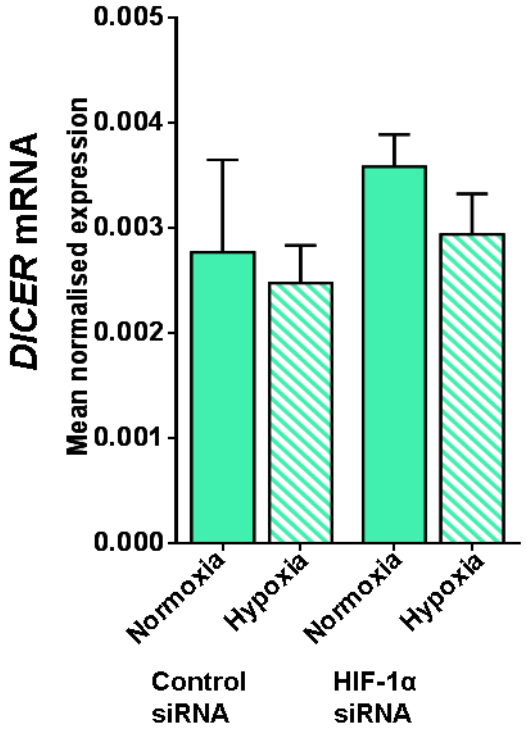

Figure 4.6 *DICER* mRNA expression in SKBR3 cells after HIF-1α suppression in hypoxia vs. normoxia.

DICER mRNA levels were analysed by real time RT-PCR and normalised to 18S ribosomal RNA levels. *DICER* mRNA expression in SKBR3 cells after transient transfection with HIF-1α targeting siRNA or control siRNA, then exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia. Data represent normalized mean ±S.E (error bars) (n=3).

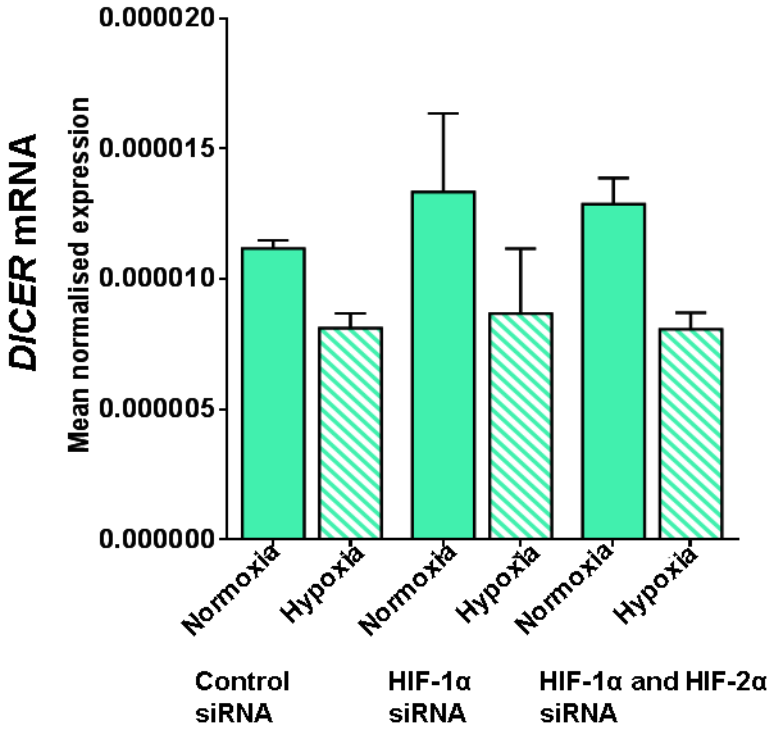

Figure 4.7 *DICER* mRNA expression in MCF7 cells after HIF-1α and HIF-2α suppression in hypoxia vs. normoxia.

DICER mRNA levels were analysed by real time RT-PCR and normalised to 18S ribosomal RNA levels. *DICER* mRNA expression in MCF7 cells after transient transfection with HIF-1α and HIF-2α targeting siRNAs or control siRNA, then exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia. Data represent normalized mean ±S.E (error bars) (n=3).

Similarly HIF-1α inhibition had no effect on DICER protein repression in hypoxia. When HIF-1α was inhibited in SKBR3 cells using siRNAs and exposed to hypoxia vs. normoxia, there was no effect on DICER protein repression (Figure 4.8).

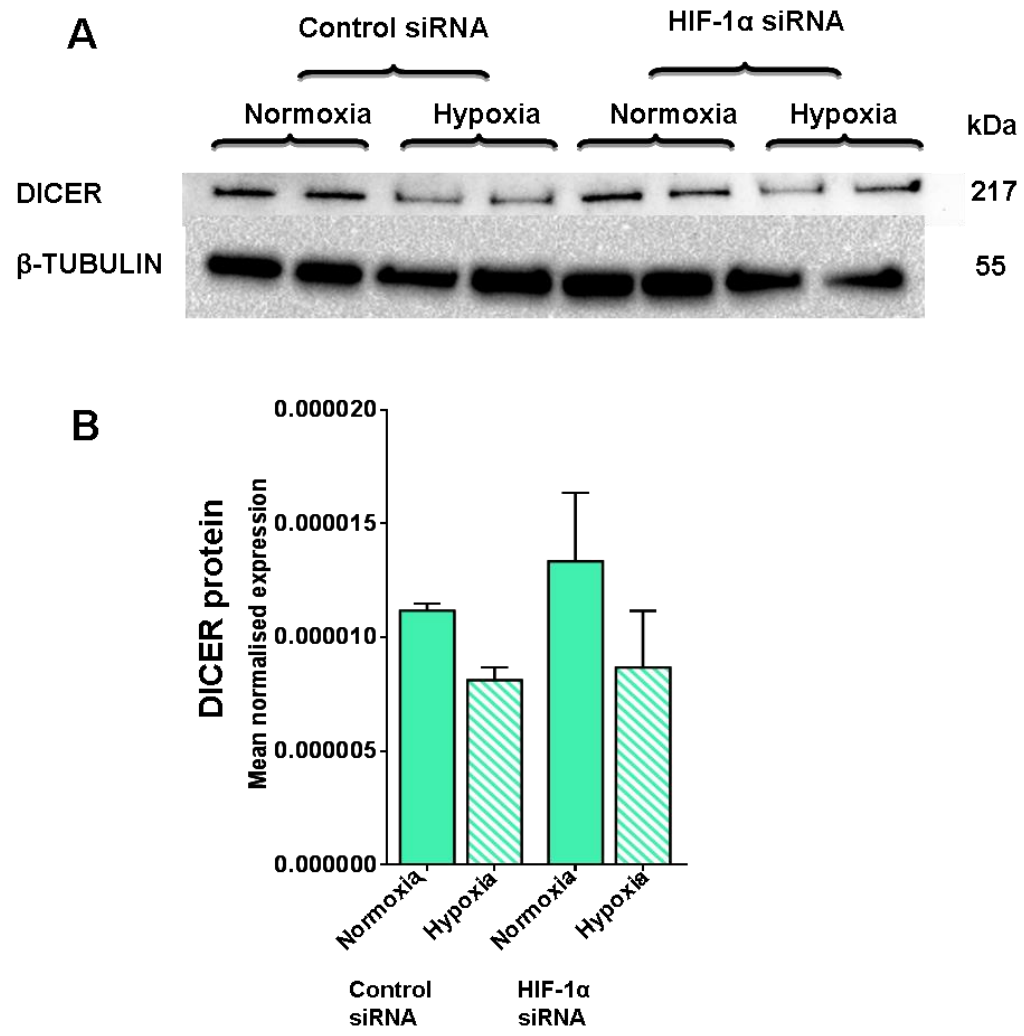

Figure 4.8 DICER protein levels in SKBR3 cells after HIF-1α inhibition in hypoxia vs. normoxia.

DICER and β-tubulin protein levels were examined by immunoblotting. A, DICER protein levels in SKBR3 cells after transient transfection with a HIF-1α targeting siRNA or control siRNA, then exposure to hypoxia (0.1% O_2 for 24 h) vs. normoxia. B, Densitometric analysis following normalisation with β-tubulin levels. Data represent normalized mean ±S.E (error bars) (n=3).

4.3.3 DICER mRNA and protein levels after HIF overexpression

To further understand the involvement of HIF in DICER repression in hypoxia the two main isoforms HIF-1α and HIF-2α were over-expressed in cells and the subsequent effect on DICER expression was examined. The full length HIF-1α and HIF-2α proteins are regulated by oxygen dependent prolyl hydroxylases. To over-express HIF-1α and HIF-2α proteins, even in normoxia, the two prolines targeted by hydroxylases were mutated to alanines when constructing these plasmids (Bracken et al., 2006). Cells were transiently transfected with plasmids containing full length HIF-1α (hHIF-1α P402A/P564A) (Bracken et al., 2006), HIF-2α (hHIF-2α P405A/P531A) (Bracken et al., 2006) or control plasmid (pcDNA 3.1 vector), and then exposed to hypoxia (0.1% O_2 for 48 h) vs. normoxia. HIF-1α protein levels were examined by immunoblotting to confirm HIF over-expression. HIF-1α protein levels showed a consistent increase after HIF over-expression in normoxia when compared to the control (Figure 4.9). However this was lower than HIF levels observed after exposure to hypoxia.

After seeing that HIF-1 levels were induced in normoxia with the HIF-1 over expression plasmids, the effects of HIF-1 over expression on *DICER* mRNA and protein levels was examined. There was no effect of HIF over-expression in normoxia or in hypoxia on *DICER* mRNA levels when compared to the control (Figure 4.10). Similarly there was no effect of HIF over-expression in normoxia on DICER protein levels when compared to the control (Figure 4.11). Dicer protein levels were normalised to the total protein on the PVDF memebrane (Figure 4.11B). These observations and lack of effect of HIF siRNAs on DICER repression suggest no major role for HIF in the hypoxic regulation of DICER.

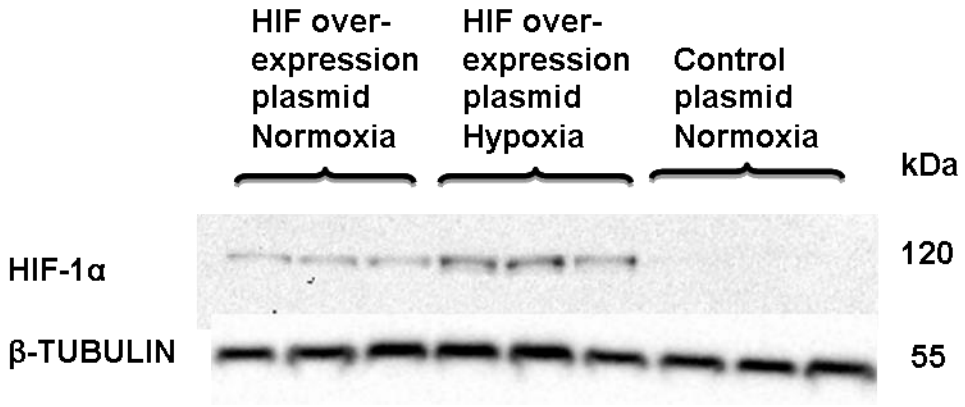

Figure 4.9 HIF-1α protein levels in SKBR3 cells after HIF over expression.

HIF-1α and β-tubulin protein levels were examined by immunoblotting. β-tubulin was used as the loading control. HIF-1α protein levels in SKBR3 cells after transient transfection with HIF-1α over expression plasmids or control plasmid, and exposure to hypoxia (0.1% O_2 for 24 h) vs. normoxia.

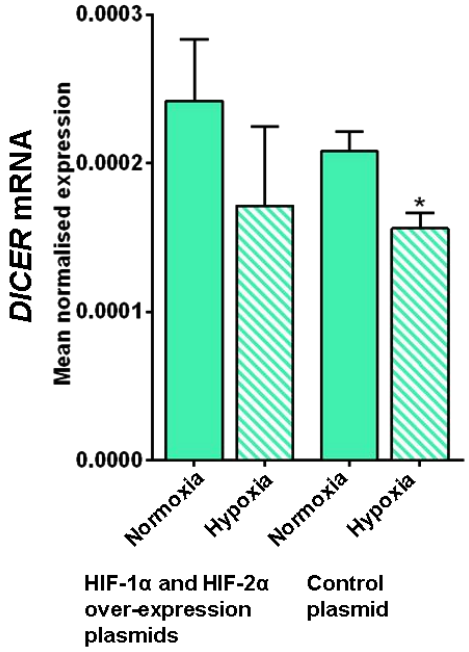

Figure 4.10 *DICER* mRNA expression in SKBR3 cells after HIF-1α and HIF-2α over expression.

DICER mRNA levels were analysed by real time RT-PCR and normalised to *β-2-microglobulin* mRNA levels. *DICER* mRNA expression in SKBR3 cells after transient transfection with a HIF-1α and HIF-2α over-expression plasmids or control plasmid DNA and exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia. Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $P < 0.05$ compared with parallel controls.

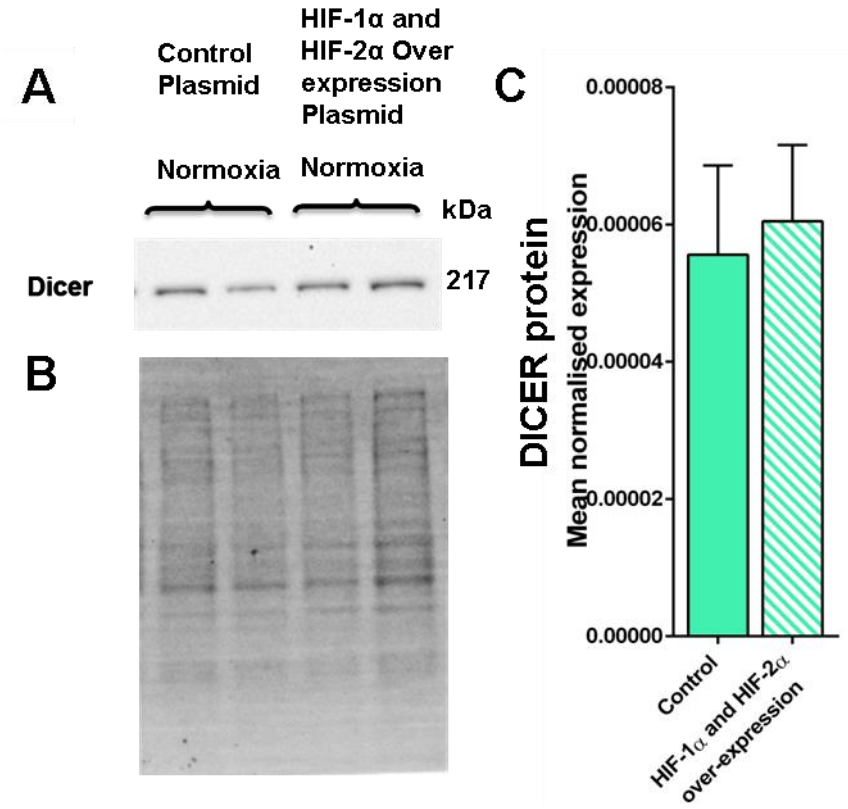

Figure 4.11 DICER protein levels in SKBR3 cells after HIF-1α and HIF-2α over expression.

DICER protein levels were examined by immunoblotting. A, DICER protein levels in SKBR3 cells after transient transfection with a HIF-1α and HIF-2α over-expression plasmids or control plasmid DNA and exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia. B, Stain-free image of SKBR3 cell lysates separated on TGX Stain-Free 'Any kD' SDS gel, transferred onto a PVDF membrane. C, Densitometric analysis following normalisation with total protein on PVDF membrane. Data represent normalized mean ±S.E (error bars) (n=3).

4.3.4 DICER mRNA and protein levels in RCC4 cells with +VHL/-VHL

The von Hippel Lindau protein (pVHL) is an essential component of the E3 ubiquitin ligase and it binds to the hydroxylated proline residues in HIF-1α and facilitates ubiquitination followed by rapid proteasomal degradation (Maxwell et al., 1999). In the absence of pVHL, due to mutations in the *VHL* gene, HIF-1α is not degraded and therefore HIF-1α accumulates and genes with HREs are transcriptionally activated (Maxwell et al., 1999, Cockman et al., 2000). In this study two RCC4 cell lines were used (detailed information in chapter 2) RCC4 +VHL cell line is a VHL deficient cell line stably transfected with a plasmid containing the *VHL* gene, while the RCC4 –VHL cell line is a VHL deficient cell line stably transfected with the empty expression vector and does not contain the *VHL* gene.

To verify the VHL status of these cell lines, HIF regulated miR-210 expression was examined in normoxia by real time RT-PCR and a large induction of miR-210 levels was observed in the RCC4 -VHL cell line compared to the RCC4 +VHL cell line (Figure 4.12).

To investigate the effect of the pVHL on DICER expression, the two RCC4 cell lines (RCC4 +VHL and RCC4 –VHL) were exposed to hypoxia (0.1% O_2) vs. normoxia for 48 h and *DICER* mRNA expression was examined by real time RT-PCR and protein levels by immunoblotting. There was no significant change in DICER mRNA levels after exposure to hypoxia (0.1% O_2 for 48 h) in the RCC4 +VHL or RCC4 -VHL cell lines (Figure 4.13A). Even the RCC4 -VHL or the cell line without the VHL gene did not show a significant down regulation of DICER in hypoxia. There was no change in DICER protein levels in hypoxia vs. normoxia in either of the

RCC4 cell lines (Figure 4.13B) further supporting the argument against a major role for HIF in the regulation of DICER protein levels by hypoxia in this cell line.

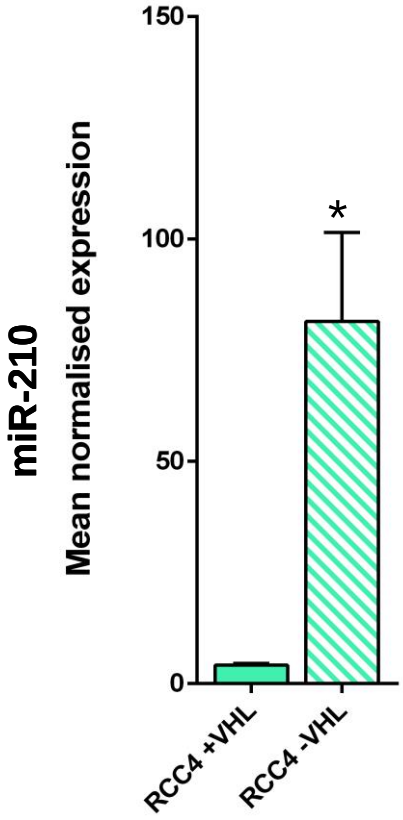

Figure 4.12 miR-210 expression in RCC4 cell lines in normoxia.

miR-210 levels were analysed by real time RT-PCR and normalised to RNU6B levels. miR-210 expression in RCC4 +VHL and RCC4 –VHL cell lines in normoxia (P=0.003). Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $P < 0.05$ compared with parallel controls.

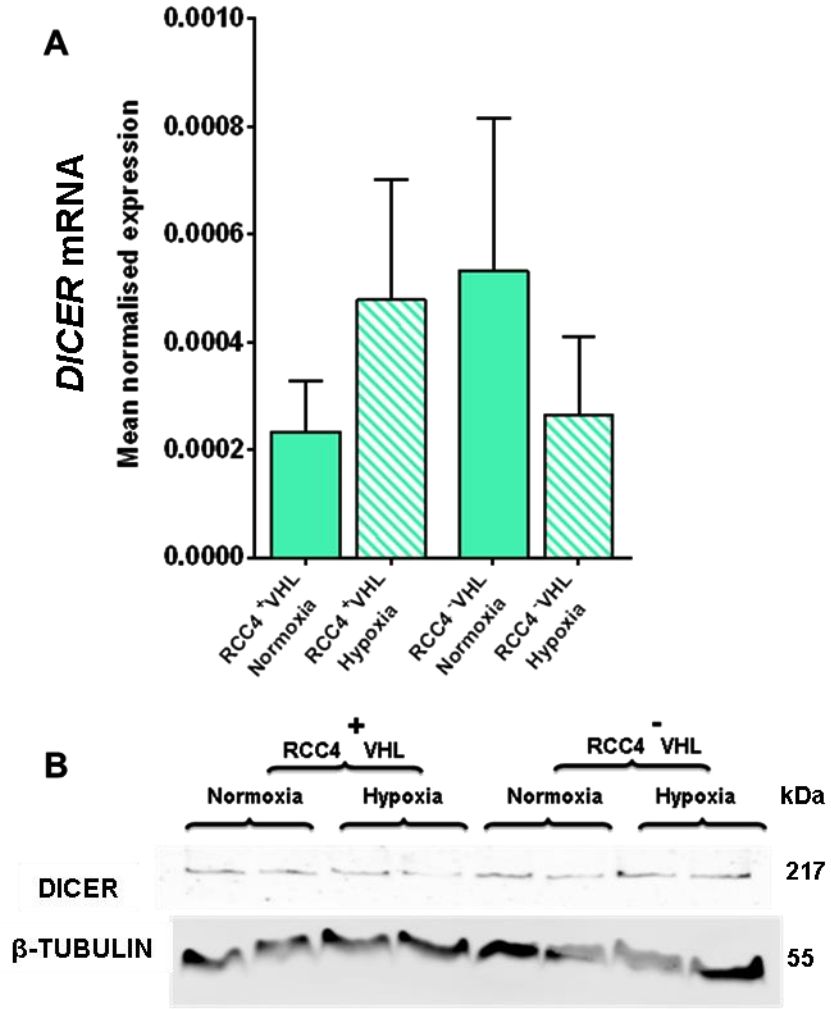

Figure 4.13 DICER expression in RCC4 +VHL and RCC4 -VHL cell lines.

DICER mRNA levels were analysed by real time RT-PCR and normalised to *β-2-microglobulin* mRNA levels. A, *DICER* mRNA levels in RCC4 +VHL/-VHL cells after exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia. DICER and β-tubulin protein levels were examined by immunoblotting. β-tubulin was used as the loading control. B, DICER protein levels in RCC4 +VHL/-VHL cells after exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia. Data represent normalized mean ±S.E (error bars) (n=3).

4.4 Prolyl hydroxylase dependent regulation of DICER

The results of this study point towards a mechanism of regulation that is oxygen and HIF hydroxylase dependent but was independent of HIF-1. Under normoxic conditions HIF-1α is hydroxylated by 2-oxoglutarate dependent prolyl hydroxylases; PHD1, PHD2 and PHD3 (Bruick and McKnight, 2001, Epstein et al., 2001). As mentioned before, during the course of this work a HIF hydroxylase (PHD2) but HIF independent pathway of repression of protein levels by hypoxia was reported in which there was PHD2 mediated suppression of eukaryotic elongation factor-2 (EEF-2) activity and protein translation under acute hypoxia (Romero-Ruiz et al., 2012). This study examined the hypothesis that PHD2 and oxygen dependent modulation of protein translation might be responsible for the DICER repression in hypoxia. SKBR3 cells were transiently transfected with siRNAs targeting *PHD2* and then DICER expression was examined in normoxia. In keeping with a role for PHD2 in the regulation of DICER expression we saw a significant decrease in *DICER* mRNA levels (P=0.0008) (Figure 4.14) and a large decrease in DICER protein levels with PHD2 suppression in normoxia (Figure 4.15). Dicer and PHD2 protein levels were normalised to the total protein on the PVDF memebrane (Figure 4.15B).

Over-expression of PHD2 and its effect on DICER repression in hypoxia was not done due to the technical difficulties involved in successfully over-expressing PHD2 in hypoxia and also PHD2 requires O_2 for its HIF hydroxylase activity. A stable cell line over-expressing PHD2 was not available during this study.

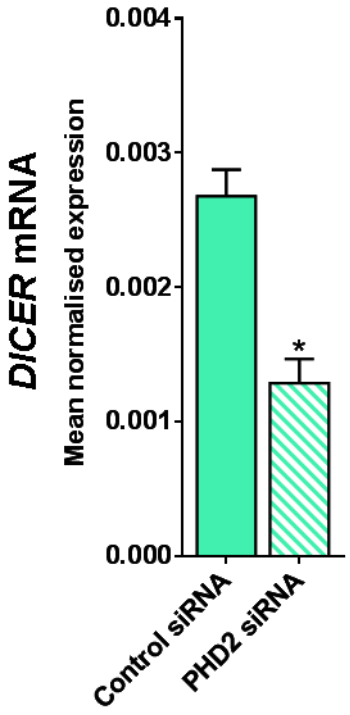

Figure 4.14 Prolyl hydroxylase-2 dependent regulation of *DICER* mRNA.

DICER mRNA levels were analysed by real time RT-PCR and normalised to *β-2-microglobulin* mRNA levels. *DICER* mRNA expression in SKBR3 cells after transient transfection with *PHD2* targeting siRNA or control siRNA in normoxia (P=0.0008). Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $P < 0.05$ compared with parallel controls.

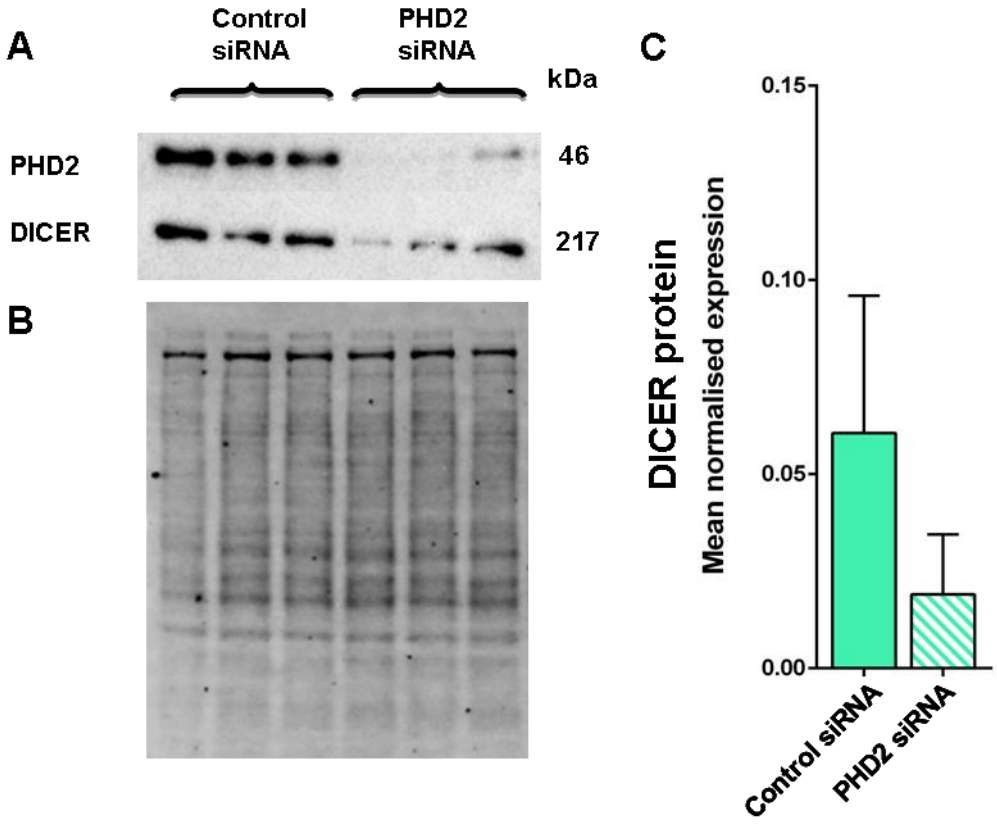

Figure 4.15 Prolyl hydroxylase-2 dependent regulation of DICER protein.

DICER and PHD2 protein levels were examined by immunoblotting. A, DICER and PHD2 protein levels after transient transfection of SKBR3 cells with siRNA targeting *PHD2* or control siRNAs, in normoxia. B, Stain-free image of SKBR3 cell lysates separated on TGX Stain-Free 'Any kD' SDS gel, transferred onto a PVDF membrane. C, Densitometric analysis followingnormalisation with total protein on PVDF membrane. Data represent normalized mean ±S.E (error bars) (n=3).

The involvement of other HIF hydroxylase enzymes (PHD1 and PHD3) or factor inhibiting HIF-1 (FIH-1) in DICER regulation was examined. PHD1 was inhibited using RNA interference with siRNAs and DICER protein levels was examined by immunoblotting. SKBR3 cells were transiently transfected with siRNAs targeting PHD1 and knock down was confirmed by immunoblotting (Figure 4.16A). Two independent siRNAs were used to suppress PHD1 levels, however only partial suppression was possible even after a double transfection with 48 h incubation with the siRNAs. The partial inhibition of PHD1 had little effect on DICER protein levels (Figure 4.16A). In keeping with previous work, PHD3 levels were very low under normoxic conditions (Appelhoff et al., 2004) and it was not possible to observe any effect of PHD3 inhibition on DICER expression. FIH-1 was inhibited in SKBR3 cells by transient transfection of siRNAs and almost complete knock down of FIH-1 was observed (Figure 4.17A). The inhibition of FIH-1 did not show any effect on DICER protein levels (Figure 4.17A).

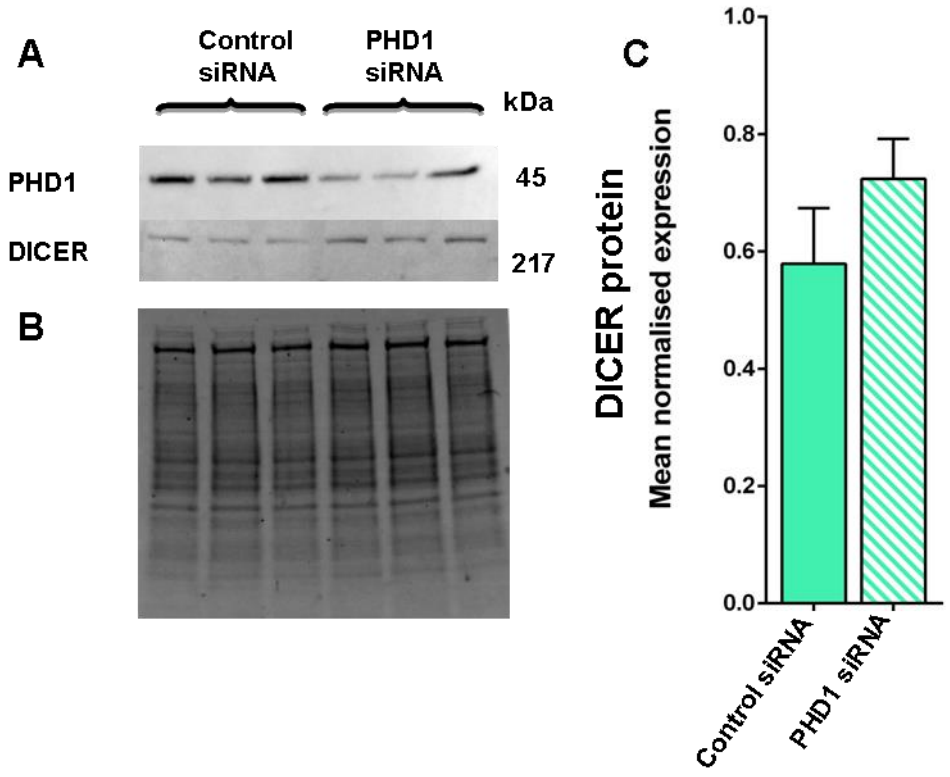

Figure 4.16 Prolyl hydroxylase-1 dependent regulation of DICER.

DICER and PHD1 protein levels were examined by immunoblotting. A, DICER protein levels after transient transfection of SKBR3 cells with siRNAs targeting *PHD1* or control siRNAs, in normoxia. B, Stain-free image of SKBR3 cell lysates separated on TGX Stain-Free 'Any kD' SDS gel, transferred onto a PVDF membrane**.** C, Densitometric analysis following normalisation with total protein on PVDF membrane. Data represent normalized mean ±S.E (error bars) (n=3).

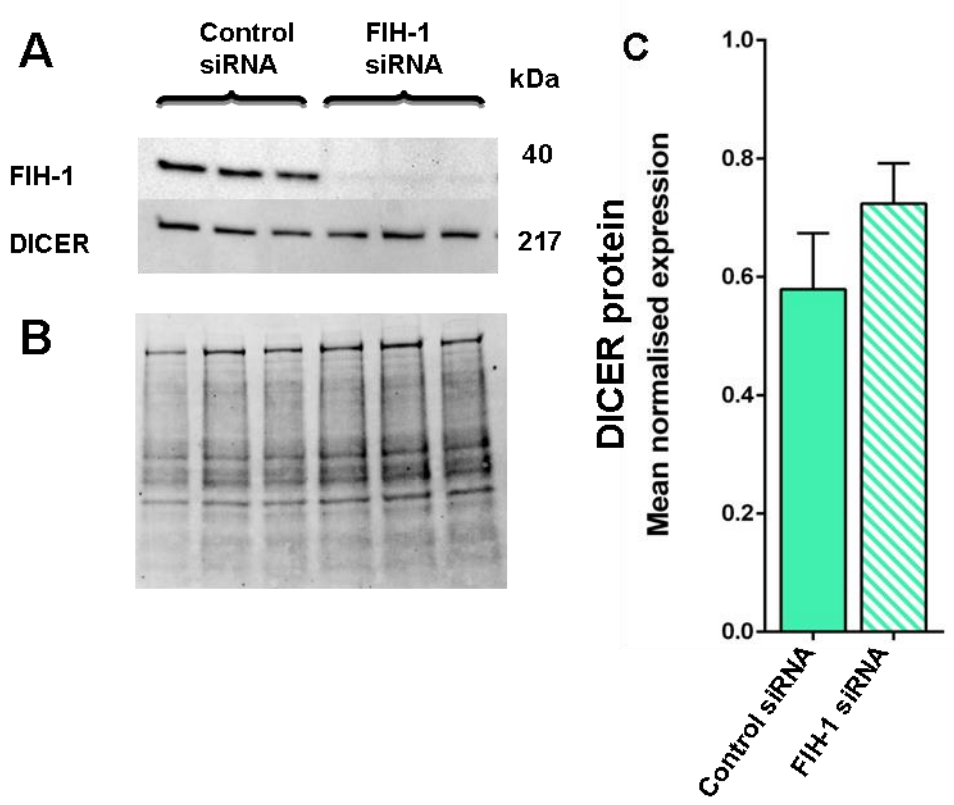

Figure 4.17 Factor inhibiting HIF-1 dependent regulation of DICER.

DICER and FIH-1 protein levels were examined by immunoblotting. A, DICER protein levels after transient transfection of SKBR3 cells with siRNAs targeting *FIH-1* or control siRNAs, in normoxia. B, Stain-free image of SKBR3 cell lysates separated on TGX Stain-Free 'Any kD' SDS gel, transferred onto a PVDF membrane**.** C, Densitometric analysis following normalisation with total protein on PVDF membrane. Data represent normalized mean ±S.E (error bars) (n=3).

4.5 MicroRNAs involved in DICER regulation

As discussed in chapter 1 some miRNAs regulate gene expression by binding to the 3'UTR of mRNAs (Bartel, 2009). DICER mRNA contains a long 3'UTR (>4000 bp) and the presence of conserved binding sites for miR-103/107 family (Martello et al., 2010) and let-7a (Forman et al., 2008) have been reported in previous studies.

The latest release from target scan (Release 6.2), a miRNA target prediction algorithm, showed several miRNA target sites on the 3'UTR of human DICER (Figure 4.18) (Lewis et al., 2005). The miRNA targets were predicted by identifying mRNAs with conserved complementarity to the seed region of the miRNA (Lewis et al., 2005). As previously mentioned, some of these predicted target sites and miRNAs, such as miR-103/107 and let-7a have been validated (Forman et al., 2008, Martello et al., 2010). It is possible that several other predicted miRNAs are also involved in regulating DICER expression.

This study examined the hypothesis that hypoxically induced miRNAs mediate the hypoxic repression of DICER expression. In addition, hypoxic regulation of miRNAs with well characterised roles in DICER regulation was also examined.

Figure 4.18 Human DICER 3' UTR.
Schematic diagram showing predicted miRNA target sires on human *DICER* 3'UTR by TargetScan release 6.2 (Friedman et al., 2009).

4.5.1 DICER regulation by miR-210

The miRNA miR-210 is the best characterised example of a miRNA that shows substantial induction under hypoxic conditions. Here the influence of manipulations of miR-210 levels on DICER expression was examined. miR-210 was over-expressed in normoxia by transient transfection of SKBR3 cells with a miR-210 mimic and a control mimic. Real time RT-PCR showed a large induction of miR-210 levels after transfection with a miR-210 mimics and there was almost no expression of miR-210 after transfection with a miR-210 antagomir (Figure 4.19). This confirmed the effectiveness of the miR-210 mimic and antagomir.

Subsequently, *DICER* mRNA and protein levels were examined after over-expressing miR-210 using the miR-210 mimic. No significant changes in *DICER* mRNA or protein levels were observed after over expressing miR-210. Only a slight decrease in *DICER* mRNA levels was observed with miR-210 over-expression in normoxia (Figure 4.20A). DICER protein levels were also slightly reduced with miR-210 over expression in normoxia (Figure 4.20B).

DICER mRNA levels showed a modest decrease after miR-210 over-expression in hypoxia and in normoxia when compared to the control (Figure 4.21). However miR-210 inhibition did not reverse the decrease in the DICER mRNA levels seen after miR-210 over expression (Figure 4.21).

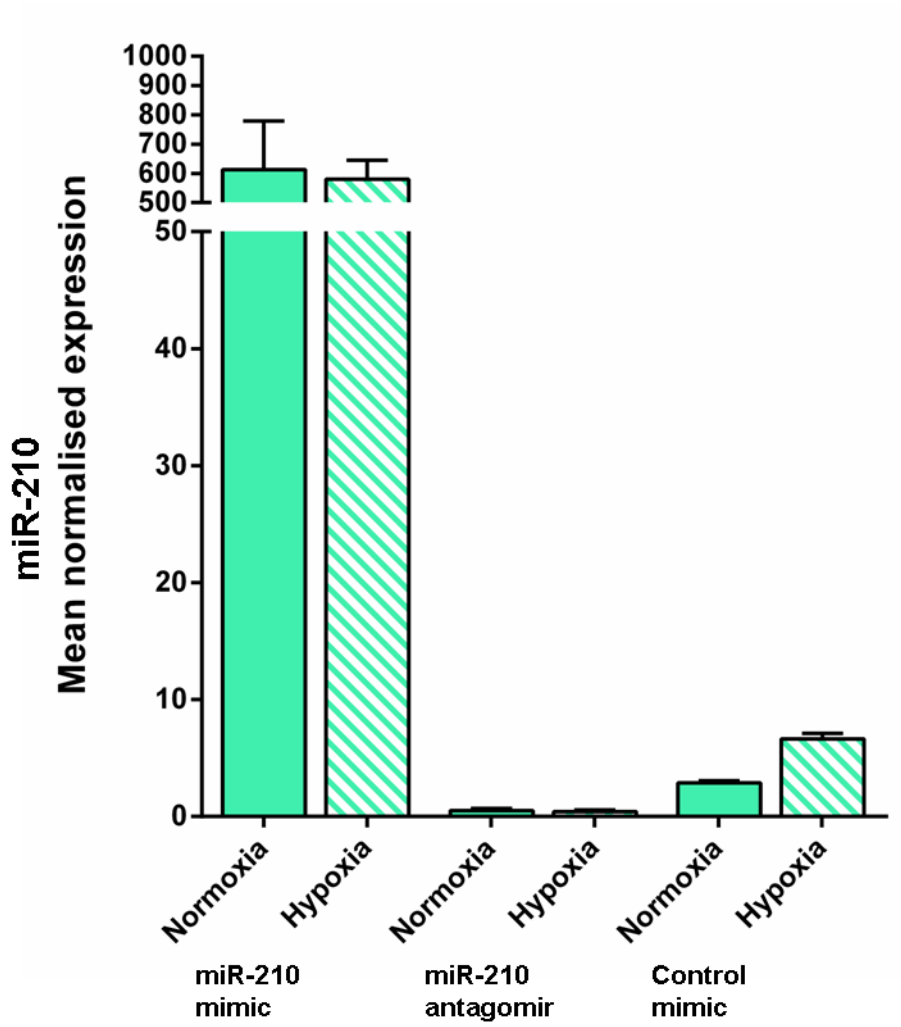

Figure 4.19 miR-210 expression after miR-210 mimic and miR-210 antagomir.

miR-210 levels were analysed by real time RT-PCR and normalised to RNU6B levels. miR-210 expression in SKBR3 cells after transient transfection with a miR-210 mimic, miR-210 antagomir or a control mimic and exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia. Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $P < 0.05$ compared with parallel controls.

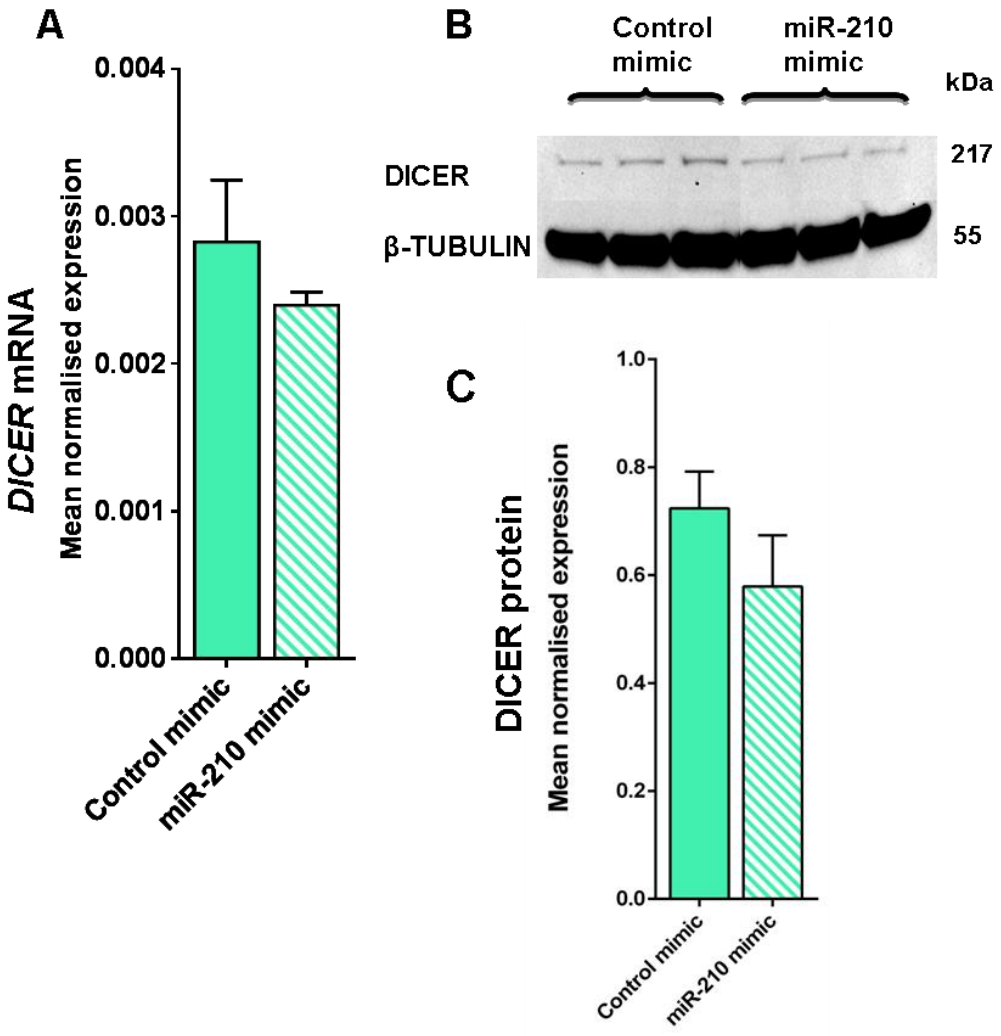

Figure 4.20 *DICER* mRNA and protein levels after over-expressing miR-210 in normoxia.

DICER mRNA levels were analysed by real time RT-PCR and normalised to *β-2-microglobulin* mRNA levels. A, I mRNA expression in SKBR3 cells after transient transfection with control mimic and miR-210 mimic in normoxia. DICER and β-tubulin protein levels were examined by immunoblotting. B, DICER protein levels in SKBR3 cells after transfection with control mimic and miR 210 mimic in normoxia. C, Densitometric analysis following normalisation with β-tubulin levels. Data represent normalized mean ±S.E (error bars) (n=3).

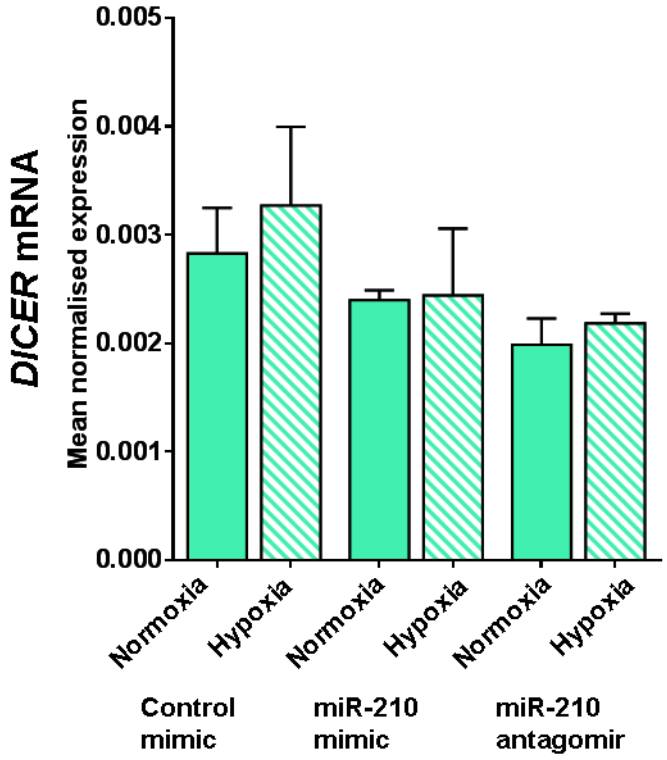

Figure 4.21 *DICER* mRNA expression after over-expressing miR-210 and inhibiting miR-210 in hypoxia vs. normoxia.

DICER mRNA levels were analysed by real time RT-PCR and normalised to *β-2-microglobulin* mRNA levels. *DICER* mRNA expression in SKBR3 cells after transient transfection with control mimic, miR-210 mimic and miR-210 inhibitor, then exposed to 0.1% O_2 for 48 h. Data represent normalized mean ±S.E (error bars) (n=3).

4.5.2 DICER regulation by miR-103 and miR-107

Two miRNAs, miR-103 and miR-107 have been shown to decrease miRNA biogenesis by targeting DICER in cancer (Martello et al., 2010) and have been reported to show hypoxic induction in some situations (Kulshreshtha et al., 2007b). This study examined miR-103 and miR-107 levels in MCF7 cells after exposure to hypoxia (0.1% O_2 for 16, 24 and 48 h) compared with normoxia to observe the effect of hypoxia on these miRNAs. MCF7 cells were used in these experiments as previous work by Kulshreshtha et al. (2007b) showed an induction of miR-103 and miR-107 in MCF7 cells. There was a modest increase in miR-103 levels after 24 h of hypoxia (0.1% O_2) and a more significant increase after 48 h of hypoxia (P=0.02) (Figure 4.22). There was an increase in miR-107 levels after 16 h (P=0.01), 24h and 48 h of hypoxia (0.1% O_2) (Figure 4.23).

To examine if the down regulation of DICER levels in hypoxia was due to repression by these two miRNAs, DICER expression was examined after miR-103/107 antagomirs and exposure to hypoxia vs. normoxia. MCF7 cells were transiently transfected with miR-103/107 antagomirs and exposed to hypoxia (0.1% O_2 for 48 h) vs.normoxia. *DICER* mRNA levels was examined through real time RT-PCR and DICER protein levels by immunoblotting. The inhibition of miR-103 and miR-107 reversed the repression of DICER mRNA levels in hypoxia (P=0.01) (Figure 4.24). Similarly, a reversal in the repression of DICER protein levels was also observed in hypoxia (P=0.002) (Figure 4.25). These observations suggest that miR-103 and miR-107 are involved in DICER regulation in hypoxia.

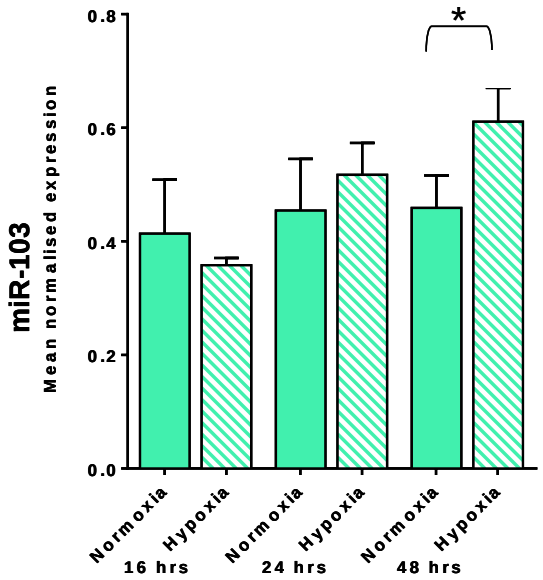

Figure 4.22 miR-103 expression in hypoxia vs. normoxia.

miRNA levels were analysed by real time RT-PCR and normalised to RNU6B levels. miR-103 expression in MCF7 cells after hypoxia (0.1% O_2 for 16, 24 and 48 h) vs. normoxia**.** Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $P < 0.05$ compared with parallel controls.

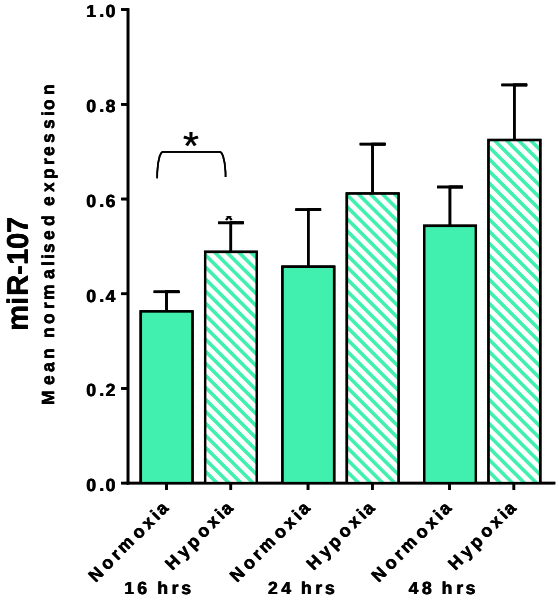

Figure 4.23 miR-107 expression in hypoxia vs. normoxia.

miRNA levels were analysed by real time RT-PCR and normalised to RNU6B levels. miR-107 expression in MCF7 cells after hypoxia (0.1% O_2 for 16, 24 and 48 h) vs. normoxia. Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $P < 0.05$ compared with parallel controls.

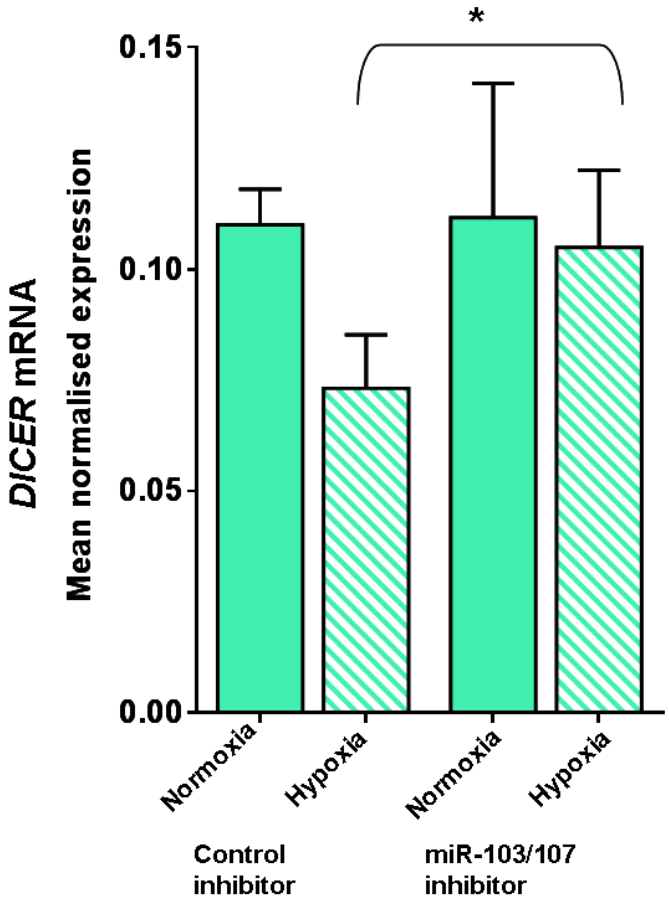

Figure 4.24 *DICER* mRNA expression after inhibiting miR-103 and miR-107 in hypoxia vs. normoxia.

DICER mRNA levels were analysed by real time RT-PCR and normalised to *β-2-microglobulin* mRNA levels. *DICER* mRNA expression after transient transfection of MCF7 cells with miR-103/107 inhibitors or control inhibitors and exposure to hypoxia (0.1% O_2 for 48 h) *vs.*normoxia (P=0.01). Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $P < 0.05$ compared with parallel controls.

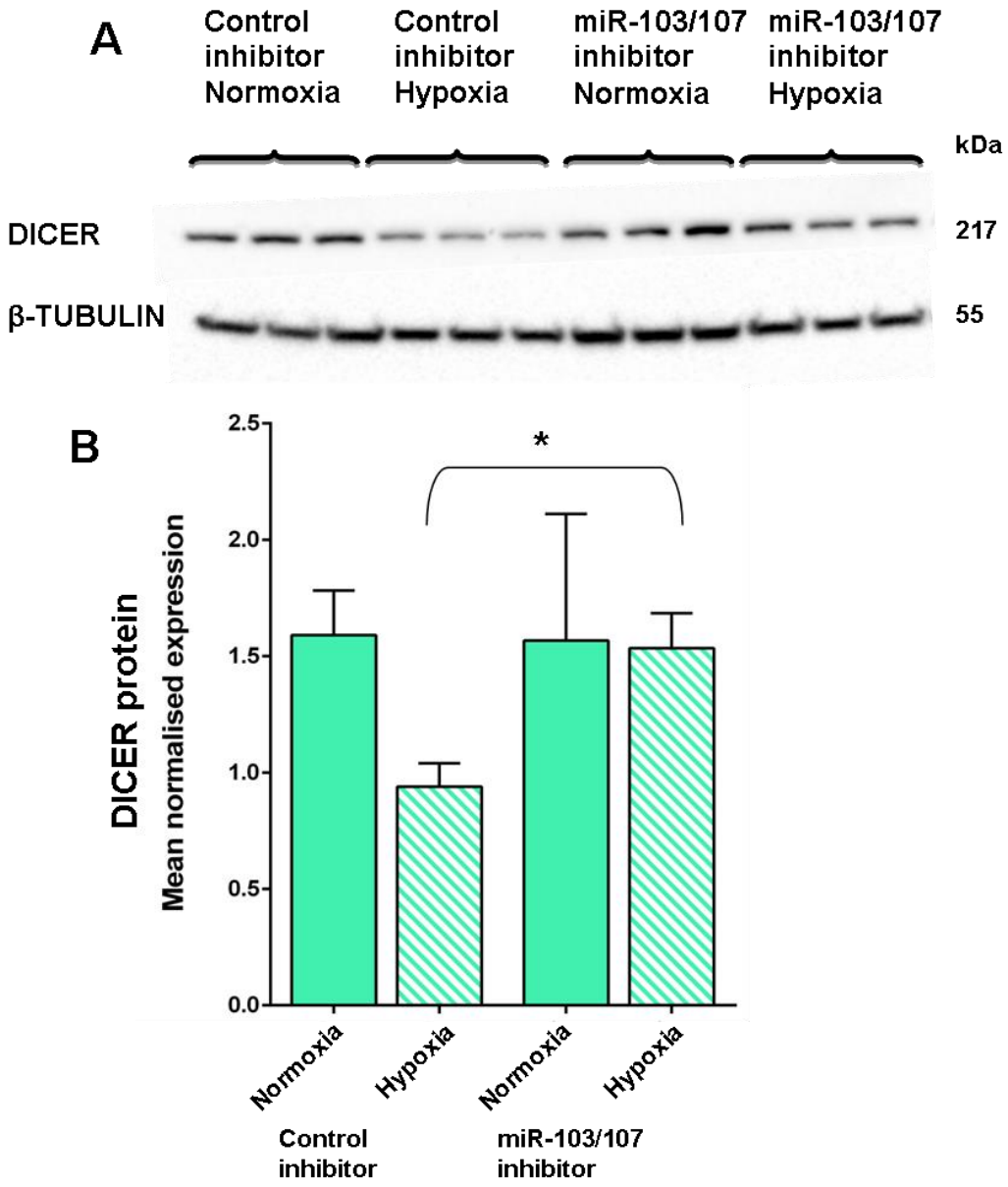

Figure 4.25 DICER protein levels after inhibiting miR-103 and miR-107 in hypoxia vs. normoxia.

DICER and β-tubulin protein levels were examined by immunoblotting. A, DICER protein levels after transient transfection of MCF7 cells with miR-103/107 inhibitors or control inhibitors and exposure to hypoxia (0.1% O_2 for 48 h) *vs.*normoxia. (P=0.002). B, Densitometric analysis following normalisation with β-tubulin levels. Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $P < 0.05$ compared with parallel controls.

4.5.3 DICER 3'UTR regulation in hypoxia

To investigate the general effect of miRNA mediated regulation of DICER expression, the effect of hypoxia on the expression of a luciferase construct with *DICER* 3'UTR was examined. In this construct the CMV promoter drives constitutive transcription of a chimeric mRNA containing the firefly luciferase coding sequence fused to the full length *DICER* 3'UTR (Lux-DICER-3'UTR) (Figure 4.26A) (Martello et al., 2010). SKBR3 cells were transiently transfected with the Lux-*DICER*-3'UTR or a control plasmid, and exposed to hypoxia (0.1% O_2 for 48 h) compared with normoxia. Cells were lysed and firefly luciferase activity was measured using a Promega luciferase kit.

This construct did not recapitulate the direction of regulation of DICER in hypoxia. There was no hypoxic repression of this construct and in fact there was a significant enhancement of luciferase activity under hypoxic conditions (Figure 4.26B).

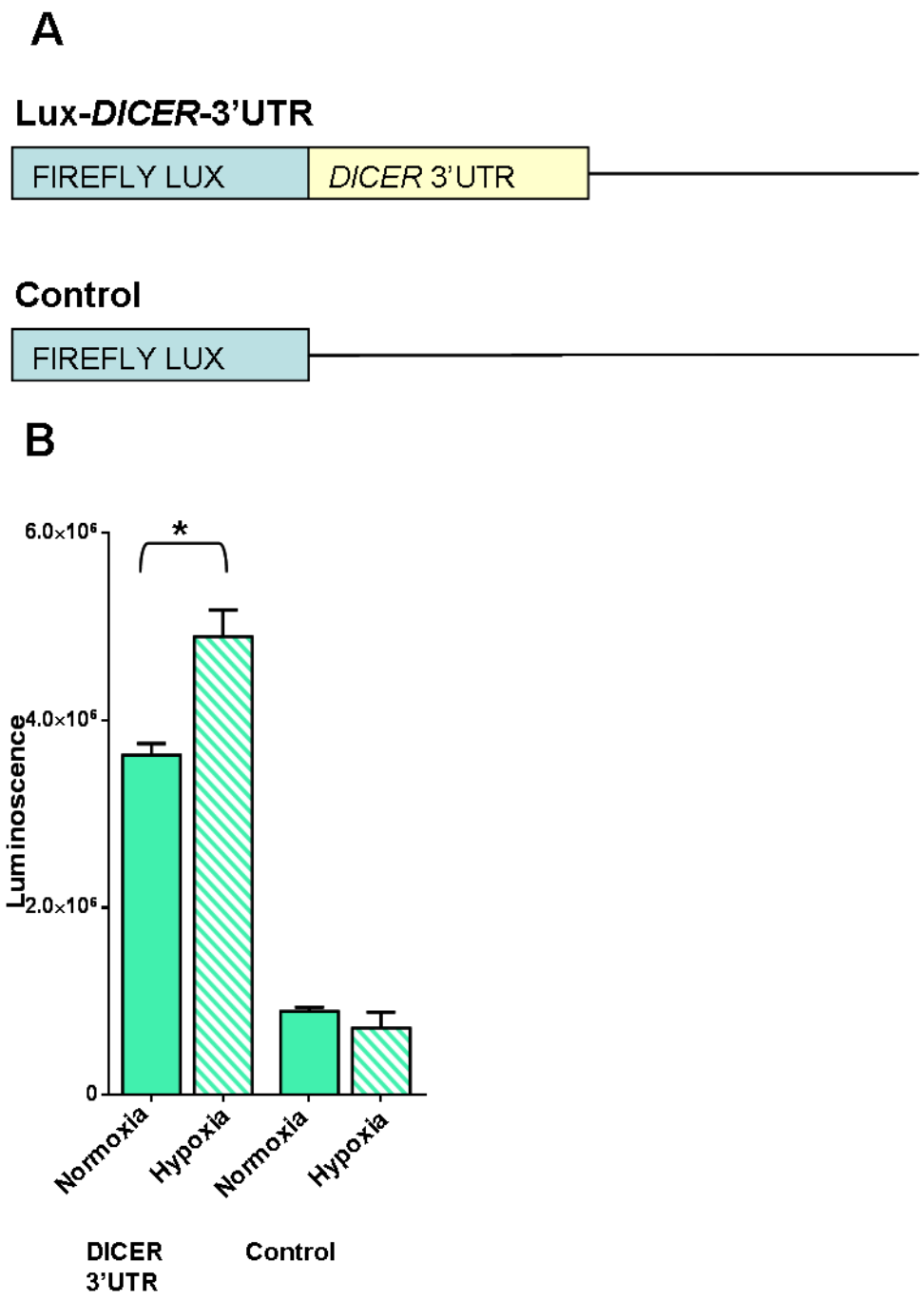

Figure 4.26 *DICER* 3'UTR not targeted by miRNAs in hypoxia.

A, Schematic representation of the reporter for miRNA activity against the *DICER* 3'UTR, containing a firefly luciferase coding sequence fused to the full length *DICER* 3'UTR (Lux-*DICER*-3'UTR). Control plasmid containing a firefly luciferase coding sequence without the *DICER* 3'UTR. B, Luciferase activity in SKBR3 cells after transfection with Lux-*DICER*-3'UTR and control plasmid, then exposure to hypoxia (0.1% O_2 24 h) vs. normoxia (P=0.03). * denotes $P < 0.05$ compared with parallel controls. Data represent mean ±S.E (error bars) (n=3).

4.6 Proteasomes in DICER regulation

Proteins in mammalian cells are mainly degraded by proteasomes present in the nucleus and the cytosol. Proteins are marked for degradation by the binding of ubiquitin molecules. The ubiquitin bound protein is then rapidly degraded by the proteasome, an ATP dependent proteolytic complex, which consists of one 20S and two 19S subunits. Proteasomal inhibitors such as Z-leu-Leu-leu-al (MG-132) readily enter cells and selectively inhibit the proteasomal degradation pathway. To investigate if proteasomes are involved in DICER protein repression in hypoxia, cells were treated with the proteasomal inhibitor MG-132 (10 μM in DMSO), and then exposed to hypoxia vs. normoxia.

HIF-1α proteins are degraded through the ubiquitin-proteasome degradation pathway (Maxwell et al., 1999). To confirm the activity of the proteasomal inhibitor (MG-132) HIF-1α protein levels were examined by immunoblotting. After treatment with MG-132 there was an increase in HIF-1α protein levels in normoxia when compared to the control in normoxia (Figure 4.27A). After MG-132 treatment, HIF-1α was stable in hypoxia (0.1% O_2) even after 48 h when compared to the control in hypoxia (Figure 4.27A). In the control, HIF-1α levels decreased after 48 h even in hypoxia, possibly due to the hypoxic induction of the PHD enzymes. These observations confirmed the proteasome inhibiting activity of MG-132.

DICER protein levels after treatment with MG-132 and exposure to hypoxia vs. normoxia was examined by immunoblotting. There was no change in DICER levels between hypoxia vs. normoxia after treatment with MG-132 (Figure 4.27A). However the control (DMSO only) treatment failed to show the decrease in DICER protein levels in hypoxia as previously observed, making it difficult to interpret these

results. The proteosomal inhibition had no effect on the DICER repression in hypoxia.

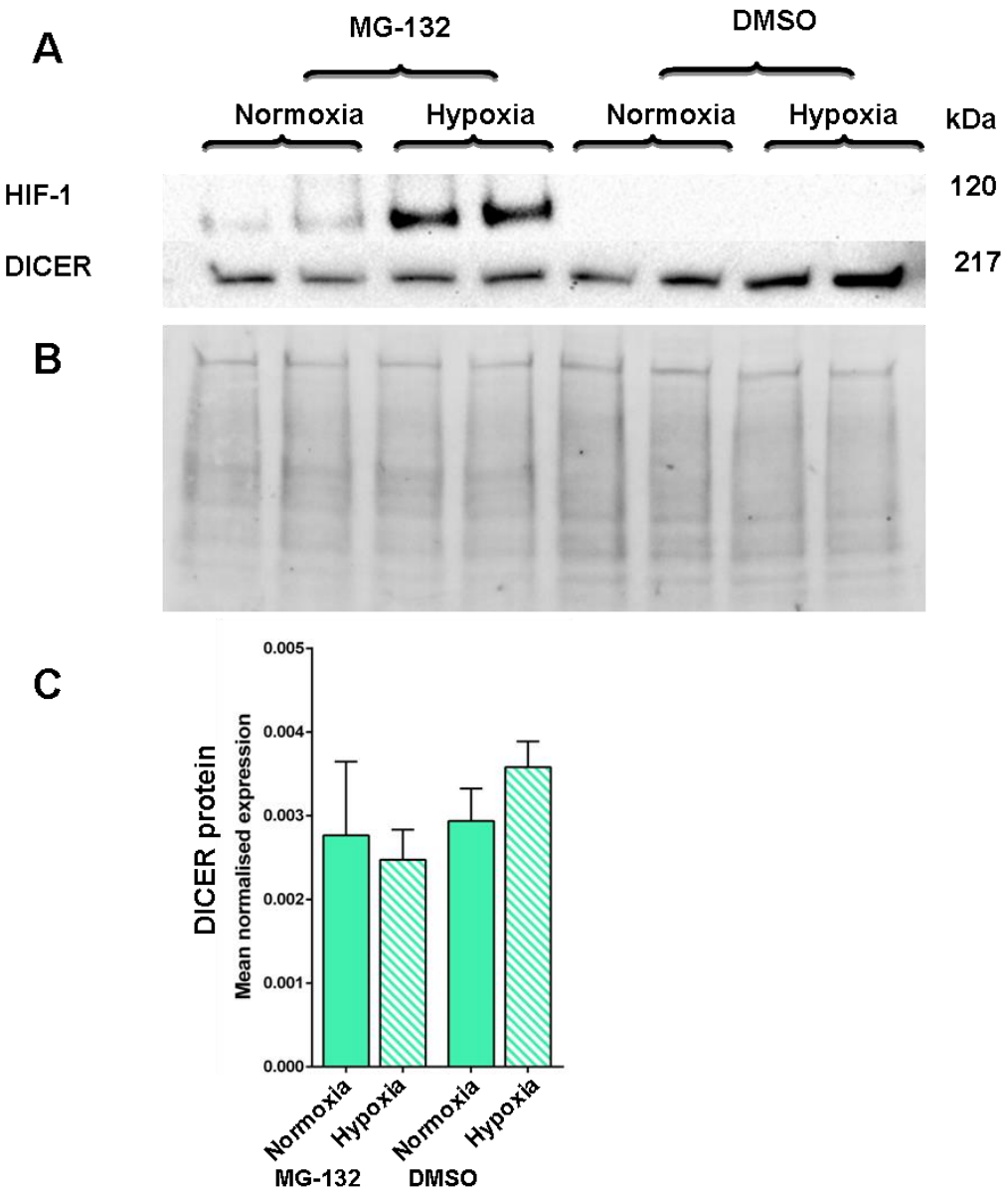

Figure 4.27 DICER regulation by proteasomes.

DICER and HIF-1α protein levels were examined by immunoblotting. A, HIF-1α and DICER protein levels in SKBR3 cells after treatment with proteasomal inhibitor MG-132 (10 μM in DMSO), and then exposed to hypoxia vs. normoxia. B, Stain-free image of SKBR3 cell lysates separated on TGX Stain-Free 'Any kD' SDS gel, transferred onto a PVDF membrane. C, Densitometric analysis following normalisation with total protein on PVDF membrane. Data represent normalized mean ±S.E (error bars) (n=3).

4.7 Discussion

The aim of this chapter was to investigate the mechanisms involved in DICER regulation in hypoxia. The effect of hypoxia on *DICER* mRNA and protein levels at transcriptional, post-transcriptional, translational and protein degradation were investigated.

HIF plays a central role in the transcriptional response to hypoxia and the HIF transcriptional system plays a dominant role in gene regulation in hypoxia (Elvidge et al., 2006). However results from this study suggest that HIF was not the main regulator of DICER in hypoxia. When HIF levels were induced in normoxia with HIF hydroxylase inhibitors DMOG and DFO, a repression in DICER levels was observed. However, when HIF was inhibited using siRNAs there was no change in the DICER repression in hypoxia. Further investigations with the overexpression of HIF-1α and HIF-2α showed no change in *DICER* mRNA or protein levels. However, it is important to note that HIF induction was low with these over-expression plasmids.

In RCC4-VHL cells HIF-1 levels was increased even in normoxia, due to the VHL mutation, when compared to the RCC4+VHL cells. However there was no change in DICER expression between these cell lines. This observation further confirms the HIF independent down regulation of DICER in hypoxia. It is important to note that there was no difference in DICER expression even in the wild type RCC4+VHL cells when exposed to hypoxia vs. normoxia. Therefore, DICER expression was not regulated by hypoxia in the RCC4 + or - VHL cell lines. Both results from HIF inhibition and over expression showed that HIF was not involved in DICER

regulation in hypoxia. This was in line with previous observations by Ho et al. (2012), where they observed in HUVECs, that DICER repression in hypoxia was not dependent on HIF-2α which is the predominant HIF-α isoform in endothelial cells (Ho et al., 2012). In addition they also looked at the DICER promoter region (1500 bp upstream of the transcription start site) of the most commonly expressed DICER mRNA variant and did not find hypoxia response elements (Ho et al., 2012).

These results point towards a mechanism of regulation that is oxygen and HIF hydroxylase dependent but independent of HIF-1. So the involvement of prolyl hydroxylases on DICER down regulation in hypoxia was examined. PHD2 inhibition reduced *DICER* mRNA and protein levels, but PHD1 and FIH-1 had no effect. PHD3 regulation could not be confirmed due to technical difficulties. These observations suggest that DICER is regulated in a HIF-1 independent but PHD2 dependent manner. A previous study showed that PHD2 regulates EEF2 phosphorylation thus affecting protein translation in hypoxia (Romero-Ruiz et al., 2012), and this might explain some of the DICER protein down regulation observed during hypoxia. PHD2 is also involved in down regulating NF-κB, another transcription factor, in a HIF independent manner in hypoxia (Chan et al., 2009). Therefore PHD2 dependent DICER regulation could be HIF independent but occur through NF-κB activation. Further investigations are needed to identify other transcriptional factors involved in DICER regulation in hypoxia independently of HIF.

Another potential mechanism of gene regulation is through binding of miRNAs to the 3'UTR of mRNAs (Bartel, 2009). Previous reports identified conserved binding sites for miR-103/107 family (Martello et al., 2010) and let-7a (Forman et al., 2008) on the *DICER* 3'UTR. Hypoxically induced miR-210 had only a modest effect on

DICER regulation. Over expression of miR-210 correlated with DICER repression. However, when miR-210 was inhibited DICER repression was not reversed. As mentioned earlier there is a large number of predicted miRNA target sites on the 3'UTR of DICER (TargetScan release 6.2) (Friedman et al., 2009), therefore manipulating the levels of a single miRNA might not have a significant effect on DICER expression. Martello et al. (2010) reported that DICER dependent miRNAs (miR-103 and miR-107) decrease miRNA biogenesis by down regulating DICER in cancer (Martello et al., 2010). A feedback loop was found to exist between these two miRNAs and DICER, to maintain DICER expression at low levels, but enough to sustain tumour growth (Martello et al., 2010). In this study an increase in miR-103 and miR-107 was observed in hypoxia, and this was in-line with previous observations (Kulshreshtha et al., 2007b). When miR-103 and miR-107 were inhibited in hypoxia there was a reversal in DICER repression by hypoxia, suggesting that DICER regulation is also mediated by miR-103 and miR-107.

In addition to looking at specific miRNAs, a general effect of miRNA mediated regulation of DICER in hypoxia was examined with a luciferase construct containing the firefly luciferase coding sequence fused to the full length DICER 3'UTR (Lux-DICER-3'UTR) (Martello et al., 2010). This construct did not recapitulate the direction of regulation of DICER in hypoxia. There was no hypoxic repression of this construct and in fact there was a significant enhancement of luciferase activity under hypoxic conditions, arguing against a role for miRNAs targeting the 3'UTR of DICER in regulation of its hypoxic repression.

Proteins in mammalian cells are mainly degraded by proteasomes. To investigate if proteasomes are involved in DICER protein repression in hypoxia, cells were treated

with the proteasomal inhibitor MG-132 and then exposed to hypoxia vs. normoxia. There was no change in DICER protein levels after treatment with the proteasomal inhibitor, suggesting that proteasomes are not involved in DICER regulation in hypoxia. However the control (DMSO only) treatment failed to show the decrease in DICER protein levels in hypoxia as previously observed, making it difficult to interpret these results. It is not clear why the DMSO control did not show DICER repression in hypoxia as expected, however previous work reported that in some situations DMSO affects significant alterations in cell phenotype as well as gene expression (Pal et al., 2012). A previous study by Ho et al. (2012) showed that proteasomes did play a role in DICER regulation. They saw DICER repression being reversed in hypoxia after treatment with a proteasomal inhibitor (MG-132) in HUVECs (Ho et al., 2012).

4.8 Chapter summary

This chapter reports the possible mechanisms involved in DICER regulation in hypoxia. The role of HIF-1 in the down regulation of DICER in hypoxia was investigated. Furthermore, HIF independent, but prolyl hydroxylase dependent, mechanisms in DICER regulation in hypoxia were examined. DICER regulation by hypoxically induced miRNAs and proteasome mediated mechanisms was also examined. The results from this chapter showed that DICER is down regulated in hypoxia, through transcriptional and/or post-transcriptional mechanisms. There is a PHD2 dependent, but HIF independent, mechanism of repression of *DICER* mRNA and protein levels under hypoxic conditions. These results also provide further support for the existence of feedback mechanisms in the regulation of DICER levels in hypoxia.

Chapter 5 Hypoxic regulation of miRNA biogenesis proteins.

5.1 Introduction

As discussed in detail in chapter 1, miRNAs are transcribed as pri-miRNAs by RNA polymerase II or III. The pri-miRNA is cleaved by DROSHA into a pre-miRNA in the nucleus. Pre-miRNAs are then transported to the cytoplasm by XPO5 and further processed into a mature miRNA duplex by DICER coupled with TARBP2. The mature miRNA strand is then incorporated into the miRISC complex composed of DICER, TARBP2 and AGO2, and function as a gene regulator.

As reported in chapter 3 of this study, a significant down regulation of DICER levels was observed in cells after exposure to hypoxia. In this chapter the effect of hypoxia on some of the other main proteins involved in miRNA biogenesis such as DROSHA, DGCR8, TARBP2 and XPO5 were examined. The purpose of these investigations was to understand if the other proteins involved in the miRNA biogenesis process are also affected by hypoxia and to identify if there is co-ordinate regulation of these proteins in hypoxia. A previous microarray study also showed modest but consistent hypoxic reductions in mRNA levels of genes (*DROSHA*, *TARBP2* and *AGO2*) involved in miRNA biogenesis in MCF7 cells (Elvidge et al., 2006).

As discussed in more detail in chapter 1, some of these proteins form functional complexes such as; DROSHA and DGCR8 complex, and DICER and TARBP2 complex. Previous research by Melo at al. (2009) reported a relationship between DICER and TARBP2 proteins, where the loss of DICER affected the stability of TARBP2 protein and vice versa (Melo et al., 2009). Han et al. (2009) reported of a relationship between DROSHA and DGCR8, where DROSHA-DGCR8 complex

cleaves the hairpin structures in the *DGCR8* mRNA reducing DGCR8 levels, while the protein-protein interaction between DROSHA and DGCR8 stabilises the DROSHA protein. Bennasser et al. (2011) reported that inhibition of XPO5 increased nuclear localisation of *DICER* mRNA and therefore DICER protein levels in the cytoplasm. There was interest to identify if the decrease or destabilisation of one miRNA biogenesis protein in hypoxia affected the stability or function of the other proteins, and also to understand possible co-ordinated regulation of these proteins under hypoxia.

Here the effect of hypoxia on DROSHA, DGCR8, TARBP2, XPO5 and AGO2 were investigated. As proteins are the main functional molecules and causative forces in the cell, the changes in protein levels were compared between hypoxia and. normoxia. DICER protein levels were significantly down regulated after exposure to hypoxia (0.1% O2) for 48 h, and these other proteins were examined under the same experimental conditions for consistency.

5.2 Aims

The aims of this chapter were to identify if hypoxia regulates other miRNA biogenesis proteins such as: DROSHA, TARBP2, XPO5 and AGO2, and to understand if there was co-ordinated regulation of these proteins in hypoxia.

5.3 Hypoxic regulation of DROSHA mRNA and protein levels

To examine if DROSHA is regulated by hypoxia, cells were exposed to hypoxia or normoxia and then DROSHA mRNA and protein levels were compared. MCF7 cells

were exposed to hypoxia (0.1% O_2 for 48 h) or normoxia and DROSHA mRNA levels were examined by real time RT-PCR. There was a down regulation of DROSHA mRNA levels in hypoxia when compared to normoxia in MCF7 cells (Figure 5.1A). DROSHA protein levels were examined by immunoblotting, and a significant down regulation of DROSHA protein levels was observed in SKBR3 cells after exposure to hypoxia (0.1% O_2 for 48 h) when compared to normoxia (Figure 5.1B).

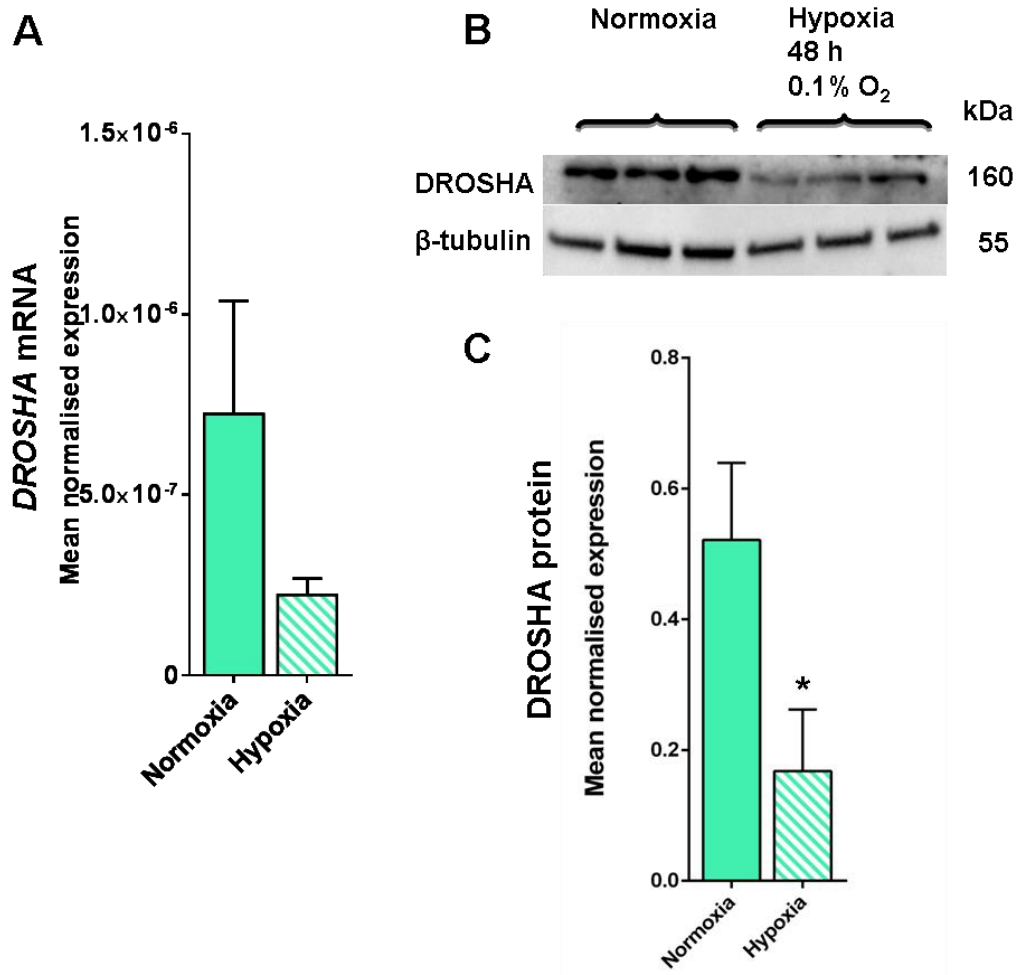

Figure 5.1 DROSHA mRNA and protein levels in hypoxia vs. normoxia.

DROSHA mRNA levels were analysed by real time RT-PCR and normalised to *β-2-microglobulin* mRNA levels. DROSHA protein levels were examined by immunoblotting. A, *DROSHA* mRNA expression in MCF7 cells after exposure to hypoxia (0.1% O2 for 48 h) vs. normoxia (P=0.05). B, DROSHA protein levels in SKBR3 cells after exposure to hypoxia (0.1% O2 for 48 h) vs. normoxia (P=0.004). C, Densitometric analysis following normalisation with β-tubulin levels. Data represent normalized mean ±S.E (error bars) (n=3). Statistical tested for by student's t-test. * denotes $P < 0.05$ compared with parallel controls.

5.4 Hypoxic regulation of DGCR8 protein levels

To examine if DGCR8 protein levels were also regulated by hypoxia, cells were exposed to hypoxia or normoxia and DGCR8 protein levels were examined by immunoblotting. There was a significant down regulation of DGCR8 protein levels in SKBR3 cells exposed to hypoxia (0.1% O_2 for 48 h) compared with normoxia (Figure 5.2).

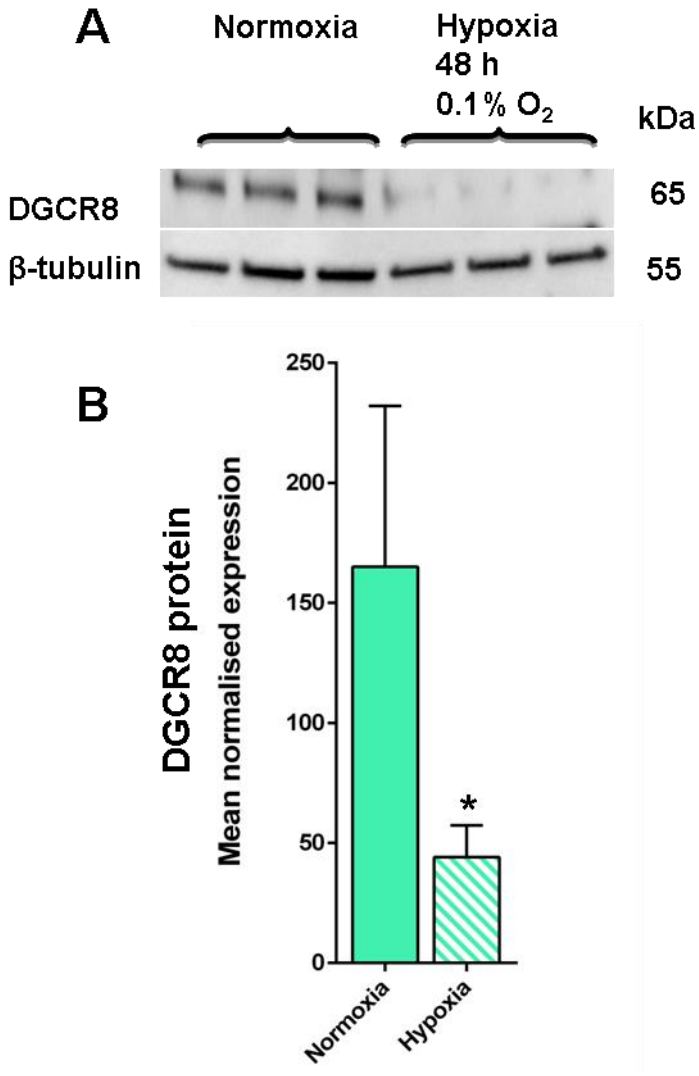

Figure 5.2 DGCR8 protein levels in hypoxia vs. normoxia.

DGCR8 protein levels were examined by immunoblotting. A, DGCR8 protein levels in SKBR3 cells after exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia (P=0.03) B, Densitometric analysis following normalisation with β-tubulin levels.

5.5 Hypoxic regulation of XPO5 protein levels

To examine if XPO5 protein levels were regulated by hypoxia, cells were exposed to hypoxia or normoxia and XPO5 protein levels were compared by immunoblotting. There was a significant down regulation of XPO5 protein levels in SKBR3 cells exposed to hypoxia (0.1% O_2 for 48 h) compared with normoxia (Figure 5.3).

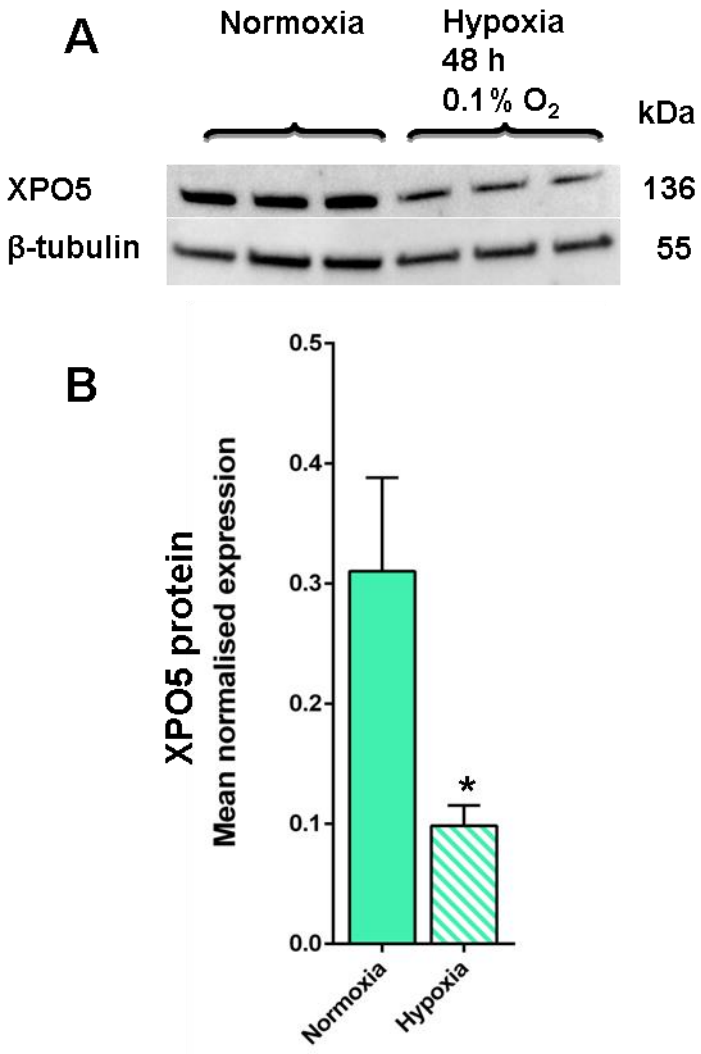

Figure 5.3 XPO5 protein levels in hypoxia vs. normoxia.

XPO5 protein levels were examined by immunoblotting. A, XPO5 protein levels in SKBR3 cells after exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia (P=0.03). B, Densitometric analysis following normalisation with β-tubulin levels. Data represent normalized mean ±S.E (error bars) (n=3). Statistical tested for by student's t-test. * denotes $P < 0.05$ compared with parallel controls.

5.6 Hypoxic regulation of TARBP2 mRNA and protein levels

To examine if TARBP2 is regulated by hypoxia, cells were exposed to hypoxia or normoxia and then *TARBP2* mRNA and protein levels were compared. MCF7 cells were exposed to hypoxia (0.1% O_2 for 48 hrs) or normoxia and TARBP2 mRNA levels were examined by real time RT-PCR. There was a significant down regulation of TARBP2 mRNA levels in hypoxia when compared to normoxia (Figure 5.4A). SKBR3 cells were exposed to hypoxia or normoxia and TARBP2 protein levels were examined by immunoblotting. There was a significant down regulation of TARBP2 protein levels in hypoxia when compared to normoxia (Figure 5.4B).

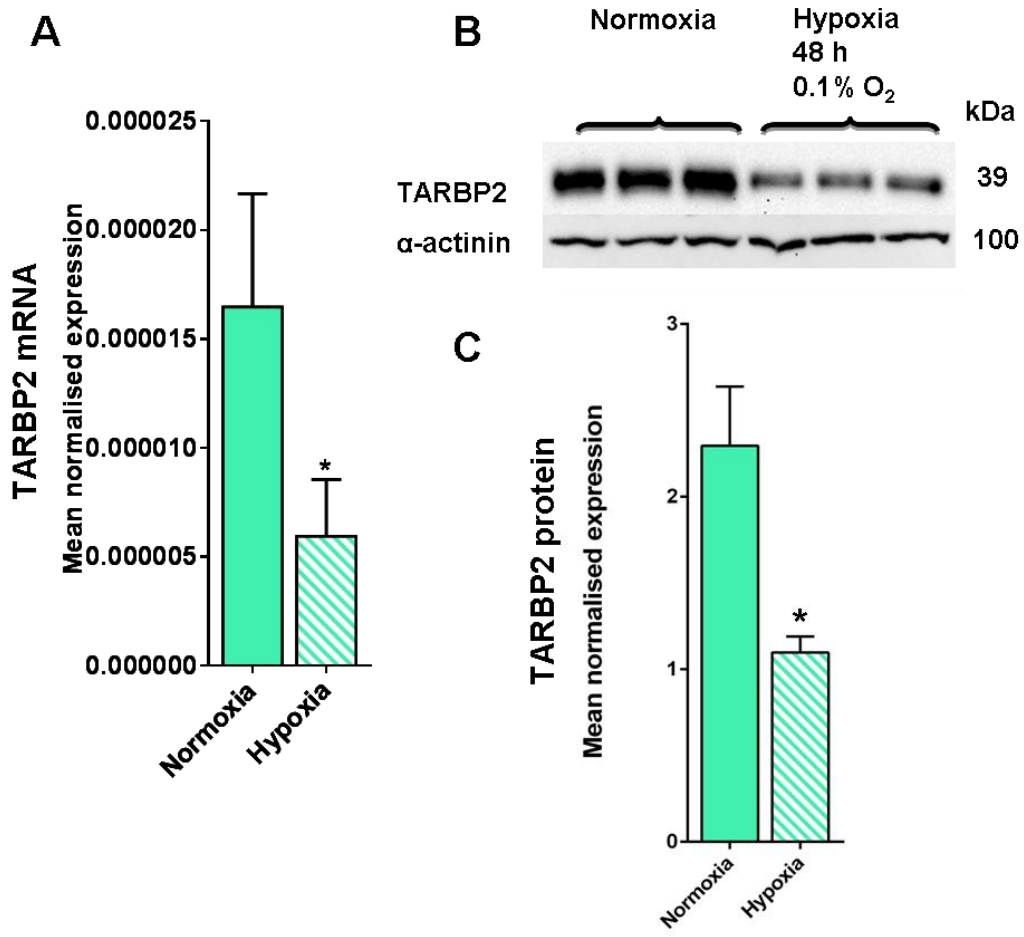

Figure 5.4 TARBP2 expression in hypoxia vs. normoxia.

TARBP2 mRNA levels were analysed by real time RT-PCR and normalised to β-2-*microglobulin* mRNA levels. TARBP2 protein levels were examined by immunoblotting. A, *TARBP2* mRNA expression in MCF7 cells after exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia (P=0.03). B, TARBP2 protein levels in SKBR3 cells after exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia (P=0.02). C, Densitometric analysis following normalisation with α-actinin levels. Data represent normalised mean ±S.E of three independent replicates (error bars) (n=3). Statistical tested for by student's t-test. * denotes $P < 0.05$ compared with parallel controls.

5.7 Hypoxic regulation of AGO2 mRNA and protein levels

To examine if AGO2 levels were regulated by hypoxia, cells were exposed to hypoxia or normoxia and then AGO2 mRNA and protein levels were compared. MCF7 cells were exposed to hypoxia (0.1% O_2 for 48 h) or normoxia and *AGO2* mRNA levels were examined by real time RT-PCR. There was a down regulation of *AGO2* mRNA levels in hypoxia when compared to normoxia (Figure 5.5A).

For detecting AGO2 protein levels by immunoblotting, a monoclonal antibody anti-AGO2 (11A9) (see chapter 2) was used. This antibody detects two bands for AGO2 in some cell types including MCF7 cells (Rudel et al., 2008). In this study two bands were detected for AGO2 on the western blot with MCF7 cell lysates. Rudel et al. (2008) suggested that this additional band migrated slightly slower than AGO2 band and this was considered a post-translational modification of AGO2. Out of the two bands detected in this study the top band showed a slight reduction in hypoxia while the second band did not show any change in MCF7 cells after exposure to hypoxia (0.1% O_2 for 48 h) when compared to normoxia (Figure 5.5B).

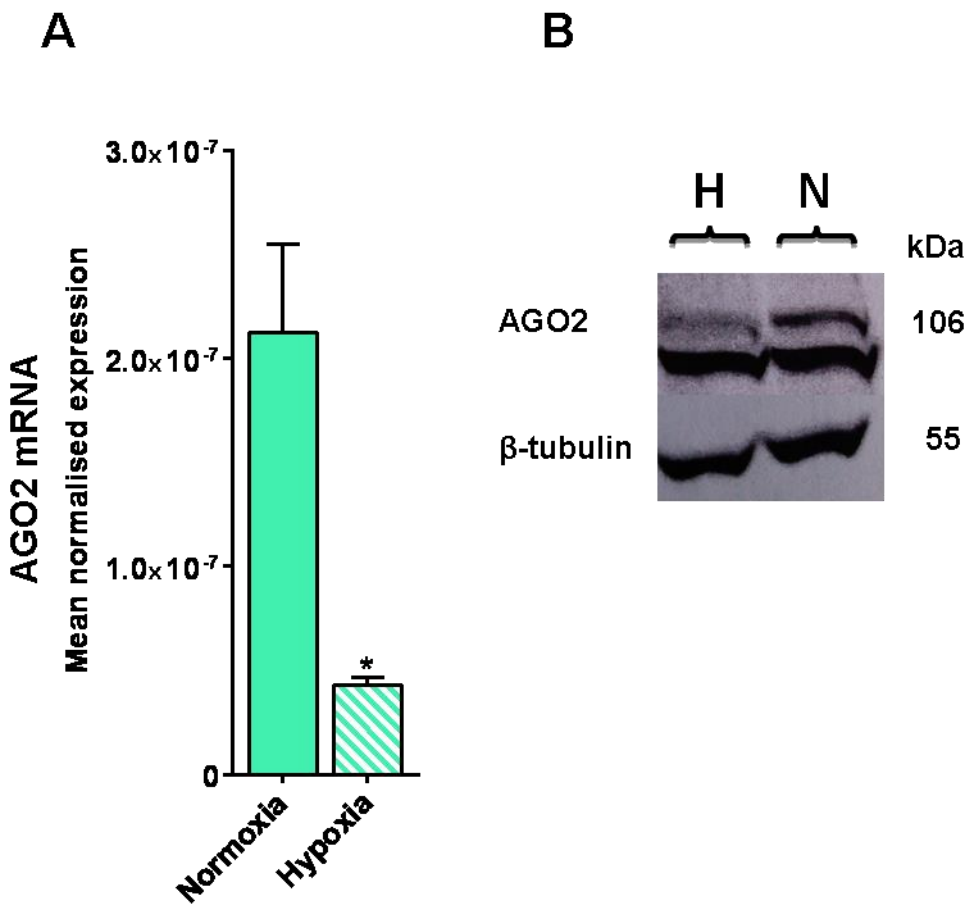

Figure 5.5 AGO2 expression in hypoxia vs. normoxia.

AGO2 mRNA levels were analysed by real time RT-PCR and normalised to 18S ribosomal RNA levels. AGO2 protein levels were examined by immunoblotting. A, *AGO2* mRNA expression in MCF7 cells after exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia (P=0.02). B, AGO2 protein levels in MCF7 cells after exposure to hypoxia (0.1% O_2 for 48 h) vs. normoxia. Data represent normalised mean ±S.E (error bars) (n=3). Statistical tested for by student's t-test. * denotes $P < 0.05$ compared with parallel controls.

5.8 Co-ordinated expression of miRNA biogenesis proteins

5.8.1 Relationship between DICER and TARBP2

Hypoxic down regulation of many proteins involved in miRNA biogenesis was observed in this study and further investigations were conducted to explore the mechanism of this effect. Previously, others have shown a powerful influence of the levels of DICER protein on the levels of TARBP2 and vice versa (Melo et al., 2009). DICER interacts with TARBP2 in processing pre-miRNAs in to mature miRNAs. Melo et al. (2009) showed that *TARBP2* mutation or impairment reduced TARBP2 protein levels and this in turn destabilised DICER protein (Melo et al., 2009).

To examine if a mechanism of co-ordinated control of protein levels might account for the parallel reduction in the levels of DICER and TARBP2 under hypoxia, the expression of each protein was inhibited using specific siRNAs and the effect on the other protein was observed by immunoblotting. *TARBP2* was inhibited in SKBR3 cells by transient transfection with siRNAs targeting *TARBP2*. The effect of this inhibition on DICER protein levels were examined by immunoblotting. TARBP2 inhibition with this siRNA was not very effective and only a modest decrease in DICER protein levels was observed after inhibiting *TARBP2* (Figure 5.6A), *DICER* was inhibited in SKBR3 cells by transient transfection using siRNAs targeting *DICER* and the effect on TARBP2 protein levels were examined. A significant decrease in TARBP2 protein levels was observed after *DICER* inhibition in SKBR3 cells (Figure 5.6B).

Protein levels of DICER and TARBP2 were examined over a time course to identify which protein changed primarily in hypoxia. DICER and TARBP2 protein levels

were examined in SKBR3 cells exposed to hypoxia (0.1% O_2) vs. normoxia for 16, 24 and 48 h by immunoblotting. A gradual decrease in TARBP2 protein levels was observed over 16, 24 and 48 h of hypoxia (0.1% O_2) (Figure 5.7). DICER protein levels were constant at 16 and 24 h of hypoxia and a significant down regulation was observed only after 48 h of hypoxia (0.1% O_2) (Figure 5.7). Therefore the decrease in TARBP2 protein levels was followed by the decrease in DICER levels.

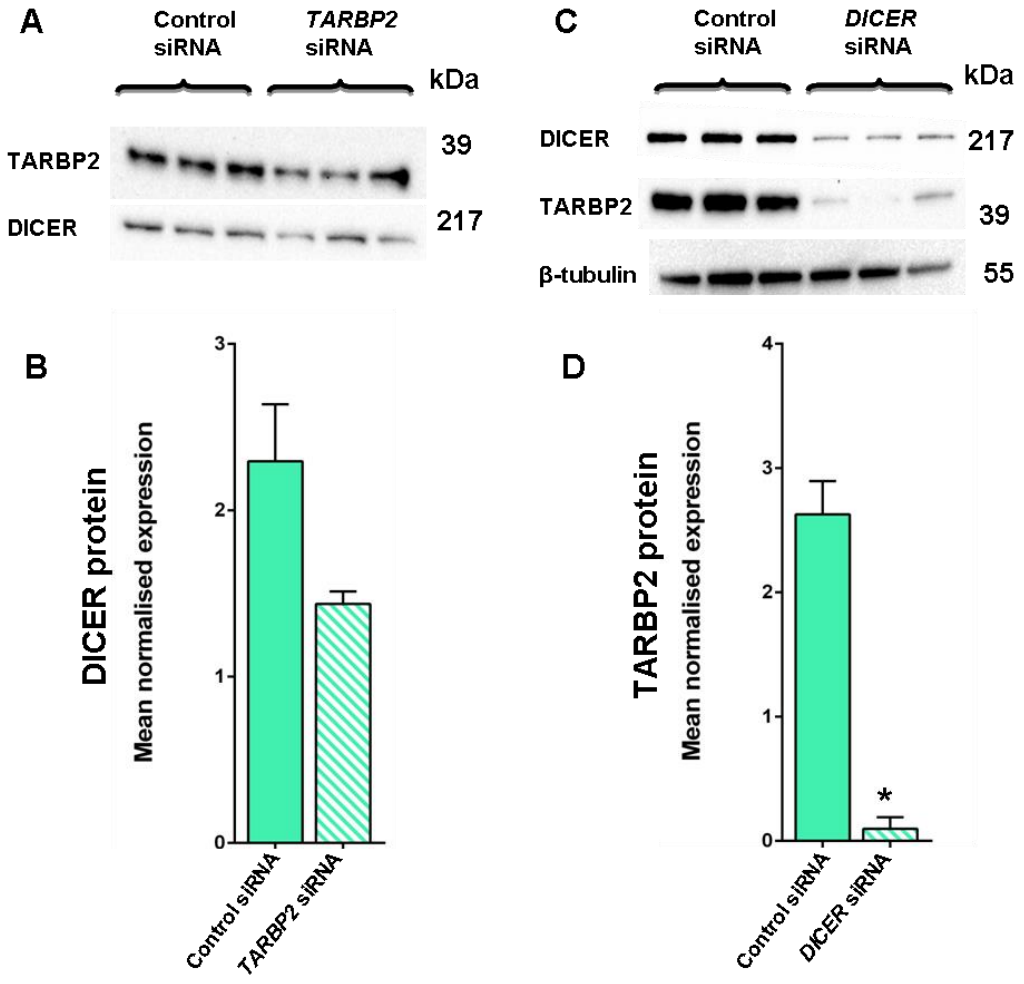

Figure 5.6 Relationship between DICER and TARBP2 protein levels.

DICER and TARBP2 protein levels were examined by immunoblotting. A, DICER protein levels in SKBR3 cells after transient transfection with siRNAs targeting *TARBP2* or control siRNAs. B, Densitometric analysis of A following normalisation with total protein on PVDF membrane. C, TARBP2 protein levels in SKBR3 cells after transient transfection with siRNAs targeting *DICER* or control siRNAs (P=0.003). D, Densitometric analysis of C following normalisation with β-tubulin levels. Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $P < 0.05$ compared with parallel controls.

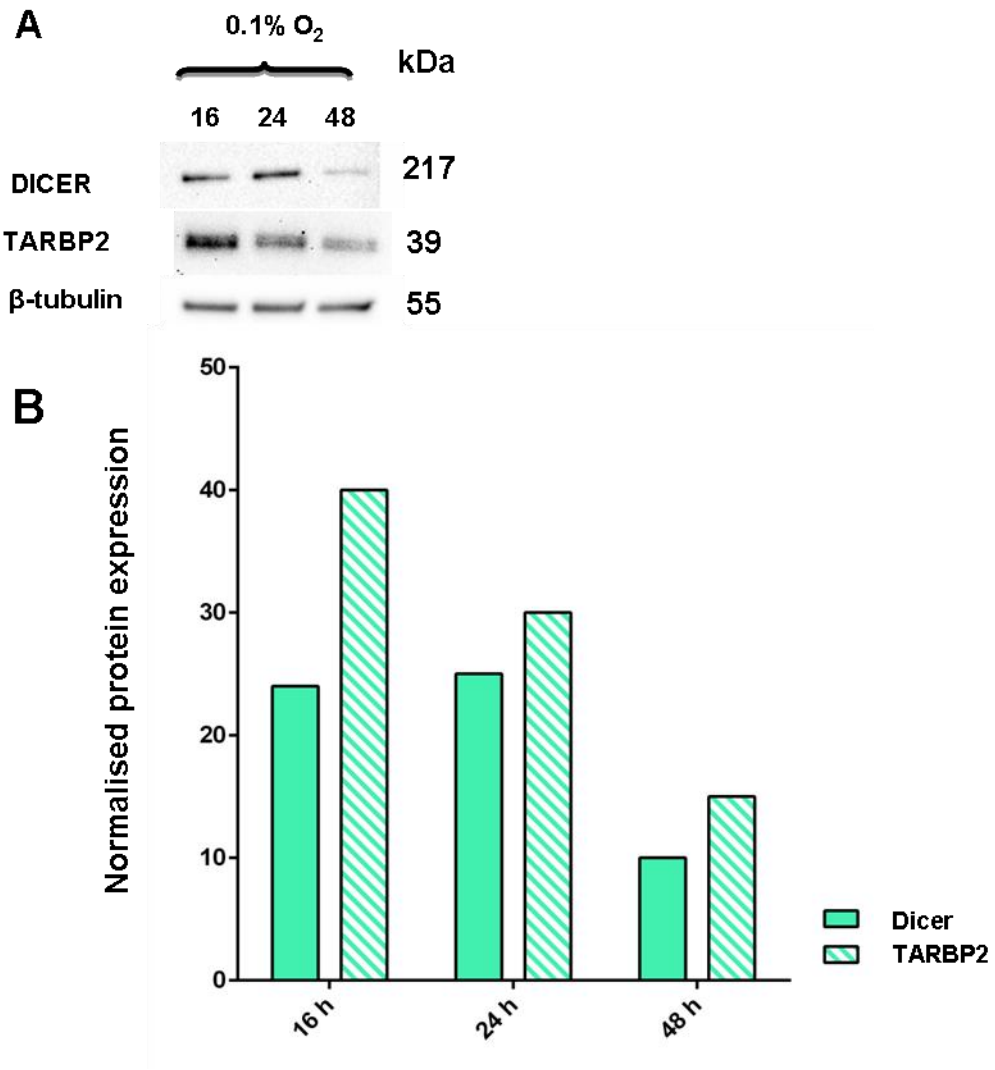

Figure 5.7 Relationship between DICER and TARBP2 protein levels.

DICER and TARBP2 protein levels were examined by immunoblotting. A, DICER and TARBP2 protein levels in SKBR3 cells after exposure to hypoxia (0.1% O_2 for 16, 24 and 48 h). B, Densitometric analysis following normalisation with β-tubulin levels.

5.8.2 Relationship between DICER and DROSHA

While investigating the relationships between DICER and other miRNA biogenesis proteins, a further relationship between DICER and DROSHA proteins was observed. DICER levels were inhibited in SKBR3 cells by transiently transfecting with siRNAs targeting *DICER* or control siRNAs, and then the effect on Dorsha protein levels was examined by immunoblotting. There was an increase in DROSHA protein levels after inhibiting *DICER* (Figure 5.8A).

After seeing a change in DROSHA protein levels with *DICER* inhibition, the effect of DROSHA inhibition on DICER protein levels was examined to further investigate the relationship between these two proteins. DROSHA levels were inhibited in SKBR3 cells by transiently transfecting with siRNAs targeting *DROSHA* or control siRNAs, and then the effect on DICER protein levels was examined by immunoblotting. A decrease in DICER protein levels was observed after DROSHA inhibition by siRNAs (Figure 5.8B).

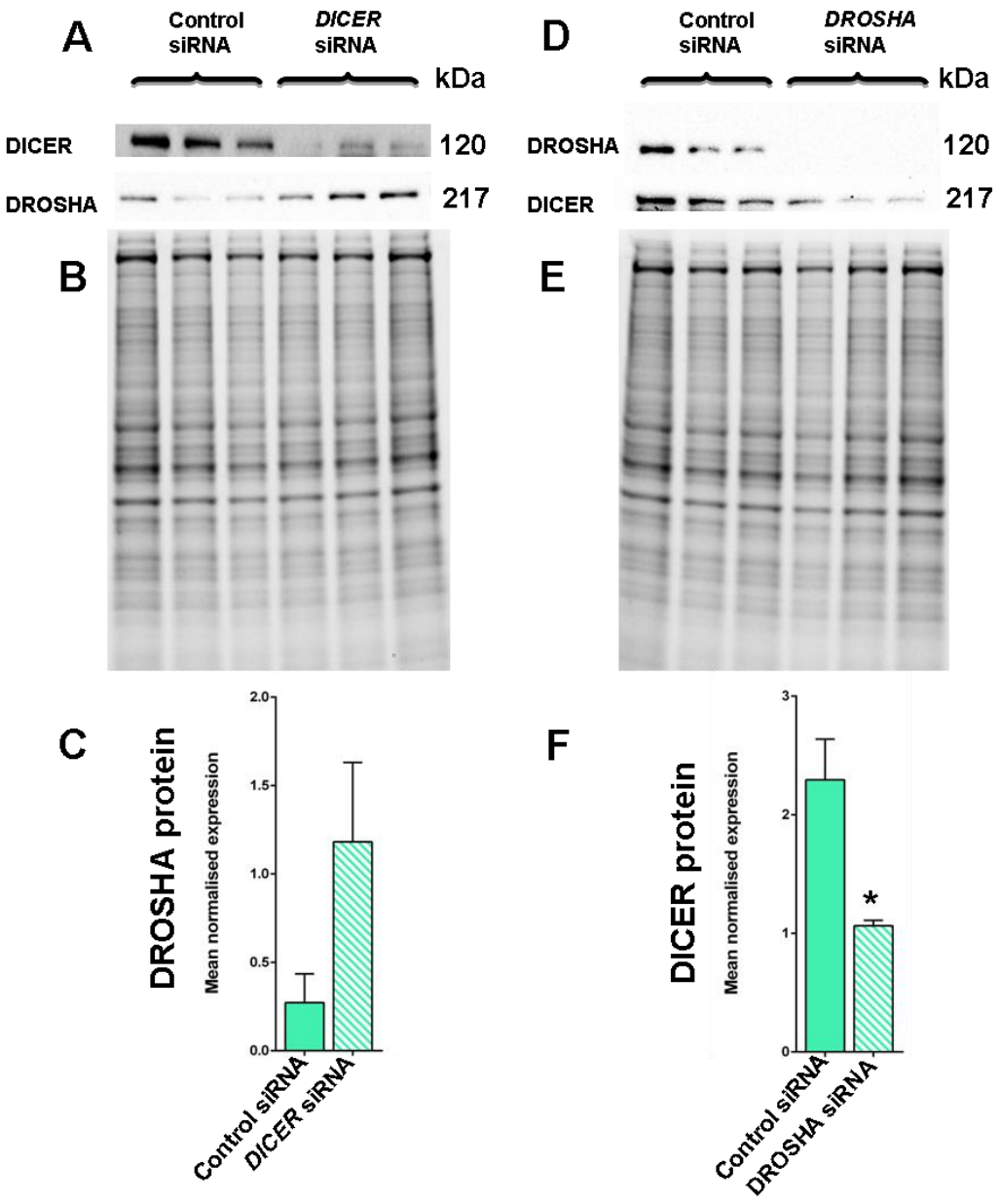

Figure 5.8 Relationship between DICER and DROSHA protein levels.

DICER and DROSHA protein levels were examined by immunoblotting. A, DROSHA protein levels in SKBR3 cells after transient transfection with siRNAs targeting DICER or control siRNAs (P=0.1). D, DICER protein levels in SKBR3 cells after transient transfection with siRNAs targeting DROSHA or control siRNAs (P=0.03). B and E, Stain-free image of SKBR3 cell lysates separated on TGX Stain-Free 'Any kD' SDS gel, transferred onto a PVDF membrane. C and F, Densitometric analysis following normalisation with total protein on PVDF membranes.

5.9 Discussion

Results from this chapter showed that the other main proteins involved in miRNA biogenesis and function, such as DROSHA, DGCR8, XPO5 and TARBP2 were also significantly affected by hypoxia. A significant down regulation of DROSHA, DGCR8, XPO5 and TARBP2 protein levels was observed in SKBR3 cells exposed to hypoxia (0.1% O_2 for 48 h), when compared to normoxia. A significant decrease in *AGO2* mRNA levels was observed but there was no significant decrease in AGO2 protein levels in hypoxia.

A few previous studies also reported of changes in important miRNA biogenesis proteins after exposure to hypoxia. Ho et al. (2012) reported a similar effect, where by DGCR8, XPO5, AGO1 and AGO2 levels decreased in HUVECs after exposure to 1% O_2 for 24 h (Ho et al., 2012). However, they did not see a down regulation of DROSHA levels in HUVECs after exposure to hypoxia (Ho et al., 2012). Wu et al. (2011) reported a different observation with regards to AGO2 protein levels in hypoxia. They observed an accumulation of AGO2 protein levels in hypoxia (Wu et al., 2011). They reported that type 1 collagen prolyl-4-hydroxylases increased in hypoxia, which led to prolyl-hydroxylation and accumulation of AGO2 (Wu et al., 2011).

Exposure to stress conditions such as hypoxia can lead to down regulation of protein synthesis as a mechanism of energy conservation in the cell (Koritzinsky et al., 2005, Wouters et al., 2005). The global down regulation of protein synthesis, resulting as a response to cellular stress, could be partly responsible for the down regulation of several proteins involved in the miRNA biogenesis pathway in hypoxia.

Another possible explanation could be that the decrease or destabilisation of one protein might have an effect on another protein, such as the relationship between DICER and TARBP2 (Melo et al., 2009). Decrease in TARBP2 levels or inactivating mutations in *TARBP2* reduced DICER protein stability (Melo et al., 2009). Results from this study showed a significant decrease in TARBP2 levels when *DICER* was inhibited and a modest decrease in DICER levels when *TARBP2* was inhibited. When the changes in DICER and TARBP2 protein levels were looked at over a time course, there was a gradual decrease in TARBP2 levels, but DICER protein levels remained almost constant up to 24 h and only showed a striking reduction at 48 h. This suggests that the decrease in TARBP2 protein levels due to hypoxia might have affected DICER protein stability, as previously shown by Melo et al (2009). Chendrimada et al. (2005) showed that knockdown of TARBP2 causes destabilisation of DICER, and vice versa (Chendrimada et al 2005). Similarly, Melo et al. (2009) also observed that TARBP2 impairment is associated with a destabilisation of DICER, and loss of TARBP2 leads to a defect in DICER activity. However, in another study they did not see destabilisation of TARBP2 when *DICER* was depleted or vice versa (Hasse et al. 2005).

Results from this study showed significant decreases in both DROSHA and DGCR8 protein levels in hypoxia. Han et al. (2009) reported that DROSHA and DGCR8 regulate each other post transcriptionally. DROSHA-DGCR8 complex cleaves the hairpin structure of *DGCR8* mRNA, reducing *DGCR8* mRNA levels, thus DGCR8 protein levels. When *DROSHA* was inhibited by siRNAs, DGCR8 protein levels increased (Han et al., 2009). DGCR8 protein stabilises DROSHA protein via a protein-protein interaction (Han et al., 2009). When *DGCR8* was inhibited by siRNAs DROSHA protein levels decreased (Han et al., 2009). It is possible that the

protein-protein interactions observed by Han et al. (2009) operate between DROSHA and DGCR8 in hypoxia and the effect of hypoxia on DGCR8 might be affecting DROSHA protein stabilisation.

In this study a relationship between DICER and DROSHA protein levels was observed. There was an increase in DROSHA protein levels after inhibiting DICER, and a decrease in DICER protein levels after inhibiting DROSHA by siRNA treatment. This was not previously reported and further studies are needed to fully understand this observation. One possible explanation could be that this regulation is mediated through miRNAs, where the decrease in DICER levels would result in a reduction in miRNAs that were involved in DROSHA regulation.

Results from this study showed a significant down regulation of XPO5 in hypoxia. A similar observation was reported by Ho et al. (2012) in HUVECs after exposure to hypoxia. Previous work showed that XPO5 is involved in DICER regulation post transcriptionally through decreasing the DICER mRNA export from the nucleus (Bennasser et al., 2011). It is possible that the decrease in XPO5, due to hypoxia, reduced DICER mRNA transport from the nucleus therefore down regulating DICER protein levels in hypoxia.

miRNAs are involved in transcriptional and post-transcriptional gene regulation (Chen and Rajewsky, 2007). Some miRNAs could be involved in regulating these miRNA biogenesis proteins, similar to the feedback loops between DICER and miRNAs (miR-103/107 and let-7a) (Martello et al., 2010, Forman et al., 2008). A recent study reported the importance of an association between mature miRNA and AGO2 protein for the stabilisation of AGO2 (Smibert et al., 2013). The loss of

miRNA biogenesis resulted in reduced mature miRNA levels, causing destabilisation of AGO2 proteins. When miRNA levels were increased artificially AGO2 protein levels elevated (Smibert et al., 2013).

5.10 Chapter summary

This chapter investigated the effect of hypoxia on some of the other main miRNA biogenesis proteins: DROSHA, DGCR8, TARBP2, XPO5 and AGO2. The changes in protein levels were compared between hypoxia and normoxia using immunoblotting techniques. Significant decreases in DROSHA, DGCR8, TARBP2 and XPO5 protein levels were observed in SKBR3 cells after exposure to hypoxia (0.1% O_2) for 48 h. *AGO2* mRNA levels significantly reduced in hypoxia but no change was observed at the protein level. DICER and TARBP2 protein levels decreased co-ordinately in hypoxia. A relationship between DICER and DROSHA was observed, where an increase in DROSHA protein levels was seen after inhibiting *DICER*, and a decrease in DICER protein levels was seen after inhibiting *DROSHA*. These observations may provide a mechanistic explanation for the reduced levels of miRNAs observed in some cancers and provides evidence for the existence of a co-ordinated mechanism of repression of miRNA biogenesis proteins in response to hypoxia.

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Chapter 6 Effects of hypoxia on miRNA expression and function

6.1 Introduction

As described in the earlier chapters, results from this study showed that hypoxia down regulates proteins with key roles in miRNA biogenesis. Significant and consistent hypoxic down regulation of DICER, DROSHA, DGCR8, TARBP2 and XPO5 proteins were observed. This chapter examined the functional consequences of the reduction of DICER and other key proteins involved in hypoxia signalling on the expression and function of miRNAs.

Several studies showed that reductions or mutations in miRNA biogenesis proteins resulted in decreased mature miRNA expression (discussed in chapter 1) (Wang et al., 2007, Ho et al., 2012, Melo et al., 2009, Iwasaki et al., 2013, Wu et al., 2012, Yi et al., 2009). Loss of DGCR8 resulted in global down regulation of mature miRNA expression in mouse embryonic stem cells and mouse skin cells (Wang et al., 2007, Yi et al., 2009). Another mouse study by Wu et al. (2012) showed that DROSHA is also essential for canonical miRNA processing and DROSHA knock out decreased the expression of many miRNAs. Furthermore, previous studies have shown that DICER inhibition using siRNAs decreased the expression of some mature miRNAs (Bu et al., 2009, Ho et al., 2012, Suarez et al., 2007). Truncating mutations in TARBP2 protein in sporadic and hereditary carcinomas with microsatellite instability showed a decreased expression of TARBP2 protein, thus a decrease in DICER stability and miRNA processing (Melo et al., 2009). XPO5 transports the pre-miRNA from the nucleus into the cytoplasm. Depletion of XPO5 levels also resulted in reduced mature miRNA levels (Yi et al., 2003, Iwasaki et al., 2013).

As previously discussed (in chapter 1) miRNAs play a key role in gene regulation (Filipowicz et al., 2008, Winter et al., 2009) and are involved in regulating many cellular processes including developmental timing, cell differentiation, cell proliferation and cell death (Brennecke et al., 2003, Xu et al., 2003).The functional mature miRNA strand is loaded into the RNA induced silencing complex (RISC) containing AGO2, by the RISC loading complex (RLC), which is composed of DICER, TARBP2 and PACT (protein activator of PKR) proteins (Gregory et al., 2005, Hammond et al., 2000, Chendrimada et al., 2005). Therefore, the depletion of these proteins may also affect the post-transcriptional gene regulation by mature miRNAs.

Previous results from this thesis showed that hypoxia down regulated proteins with key roles in the miRNA biogenesis pathway. This chapter examines the functional consequences of the reduction in DICER and other key proteins in hypoxia. For a comprehensive understanding of the global effects of hypoxia vs. normoxia on miRNA biogenesis, mature and pre-miRNA levels were compared using miRNA microarrays and real time RT-PCR techniques. To understand if this reduction in miRNA biogenesis proteins affected the miRNA mediated gene regulation through RISC assembly and function, the effect of hypoxia on the processing and gene suppression of an exogenously introduced precursor miRNA was examined. In addition a DICER activity assay was conducted to examine the influence of hypoxia on the processing of an exogenously introduced pre-miRNA.

6.2 Aims

The aim of this chapter was to investigate the effects of hypoxia on the processing, accumulation and function of miRNAs

6.3 Mature and precursor miRNA expression in hypoxia compared to normoxia

6.3.1 Mature and precursor miRNA expression analysis by microarrays

6.3.1.1 Microarray analysis after exposure to hypoxia for 16 h

As illustrated in chapters 3 and 5, hypoxia down regulates proteins involved in the miRNA biogenesis pathway. To get a more comprehensive understanding of the global affects of hypoxia compared with normoxia on miRNA biogenesis, mature and precursor miRNA levels were compared using an Affymetrix miRNA 3.1 array. Affymetrix miRNA 3.1 Array Strip consists of probe sets unique to human mature miRNA (1733) and pre-miRNA hairpins (1658). This array system was chosen as it had full miRBase coverage and contained probe sets unique to pre-miRNA hairpins.

Initially, MCF7 cells were exposed to hypoxia (0.1% O_2) or normoxia for 16 h and total RNA from these samples was analysed using the Affymetrix Genechip miRNA Array (version 3.1) system (as described in chapter 2). Each treatment consisted of three replicates. Mature and precursor miRNA expression levels were compared between hypoxia and normoxia. Intensity data were normalised using the quantile normalisation method and $\log_2$ transformed. Gene expression data were analysed using the Partek® software package. Differentially expressed genes were determined

using ANOVA and P value adjusted with FDR (False discovery rate) analysis. P<0.05 was considered as a significant change.

In keeping with the modest changes in DICER expression at this time point, no significant changes were observed in overall mature miRNA (Figure 6.1) levels in MCF7 cells exposed to hypoxia (0.1% O_2) or normoxia after 16 h.

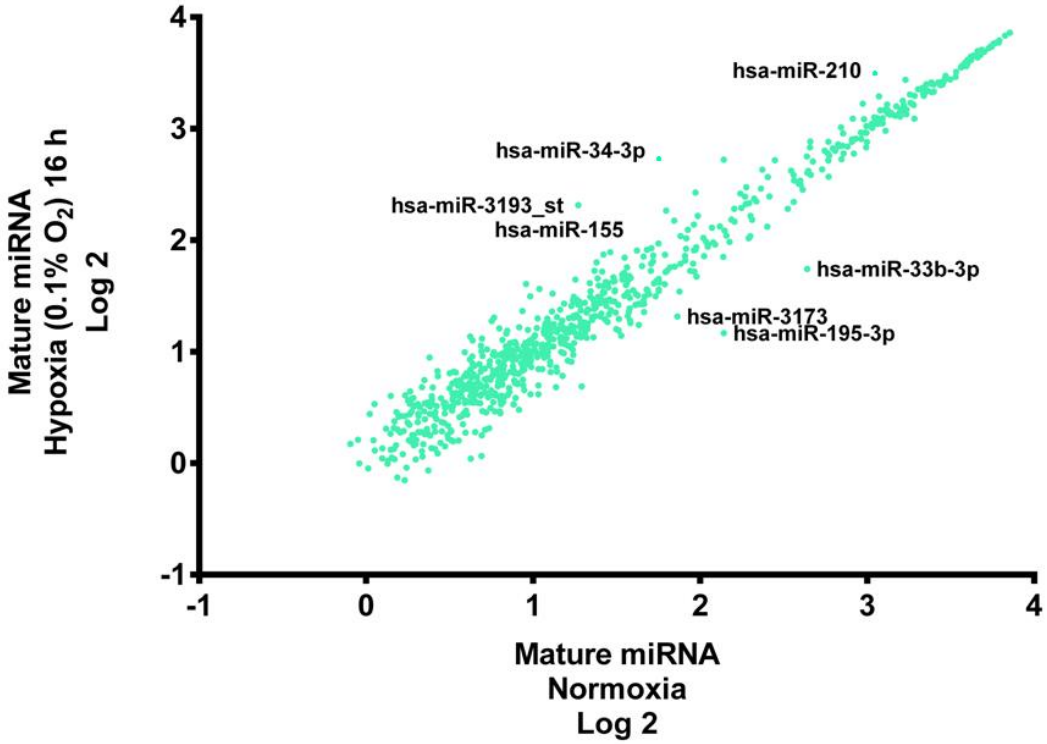

Figure 6.1 Mature miRNA expression in hypoxia (0.1% O_2 16 h) vs. normoxia.

Microarray analysis. Scatter plot of average probe intensities of mature miRNAs expressed in MCF7 cells after exposure to hypoxia (0.1% O_2) 16 h vs. normoxia (n=3).

Even though the changes in miRNA expression in hypoxia when compared to normoxia at 16 h was not statistically significant, miRNAs listed in table-6.1 were up-regulated in hypoxia with a fold change of >2, and miRNAs listed in table-6.2 were down regulated in hypoxia with a fold change of >2. Well validated hypoxically induced miR-210 (Camps et al., 2008) showed a 2.75 fold increase in hypoxia when compared to normoxia. None of these fold changes were statistically significant possibly due to variations between the replicates.

Table 6.1 miRNAs up regulated in MCF7 cells after exposure to hypoxia (0.1% O_2) vs. normoxia for 16 h.

miRNA	Fold change
hsa-miR-3193	5.90
hsa-miR-34a-3p	4.56
hsa-miR-181a-3p	2.92
hsa-miR-27a-5p	2.79
hsa-miR-210	2.75
hsa-miR-595	2.73
hsa-miR-193b-5p	2.61
hsa-miR-1825	2.53
hsa-miR-155	2.51
hsa-miR-1281	2.18
hsa-miR-4274	2.15
hsa-miR-30b-3p	2.10
hsa-miR-21-3p	2.07
hsa-miR-3185	2.03

Table 6.2 miRNAs down regulated in MCF7 cells after exposure to hypoxia (0.1% O_2) vs. normoxia for 16 h.

miRNA	Fold change
hsa-miR-33b-3p	-7.43
hsa-miR-195-3p	-4.50
hsa-miR-1979	-2.32
hsa-miR-3173	-2.22

Expression level of miR-210 in total RNA samples was examined by real time RT-PCR. A 2.5-fold induction of miR-210 levels was observed in hypoxia (0.1% O_2) when compared to normoxia after 16 h (Figure 6.2). The fold changes for miR-210 levels in hypoxia compared to normoxia were similar between the two different experimental procedures. A 2.5-fold increase in miR-210 was detected from real time RT-PCR analysis, and a 2.8-fold increase was detected from the microarray analysis.

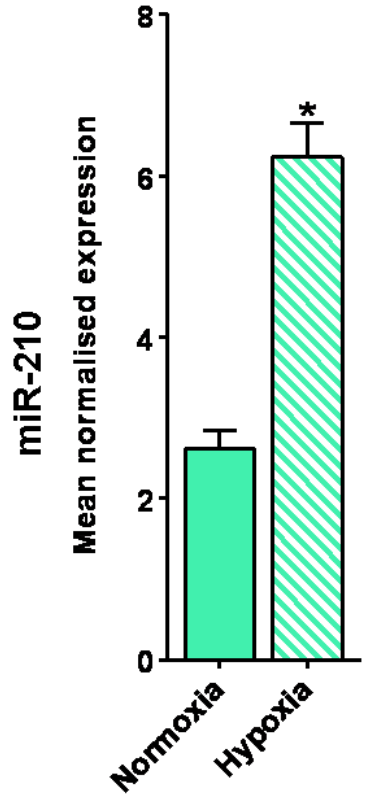

Figure 6.2 miR-210 expression in MCF7 cells in hypoxia vs. normoxia.

miR-210 levels were analysed by real time RT-PCR and normalised to RNU6B small nucleolar RNA levels. miR-210 expression in MCF7 cells after hypoxia (0.1% O_2) 16 h vs. normoxia (P=0.0004). Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $p < 0.05$ compared with parallel controls.

In addition to looking at the mature miRNA levels the changes in the hairpin (included pri-miRNAs and pre-miRNAs) levels were also examined. The changes in hairpin levels were investigated to identify if there was an accumulation of hairpins in hypoxia compared to normoxia as a result of reduced DICER processing. Hairpin (hp) intensity values produced in this microarray included both primary and precursor miRNAs. There were no significant changes in the hairpin levels in hypoxia (0.1% O_2), when compared to normoxia after 16 h (Figure 6.3). However hypoxically induced miR-210 showed an increased expression of the corresponding hp-miR-210, in keeping with previous demonstrations of transcriptional induction of miR-210 by hypoxia (Camps et al., 2008).

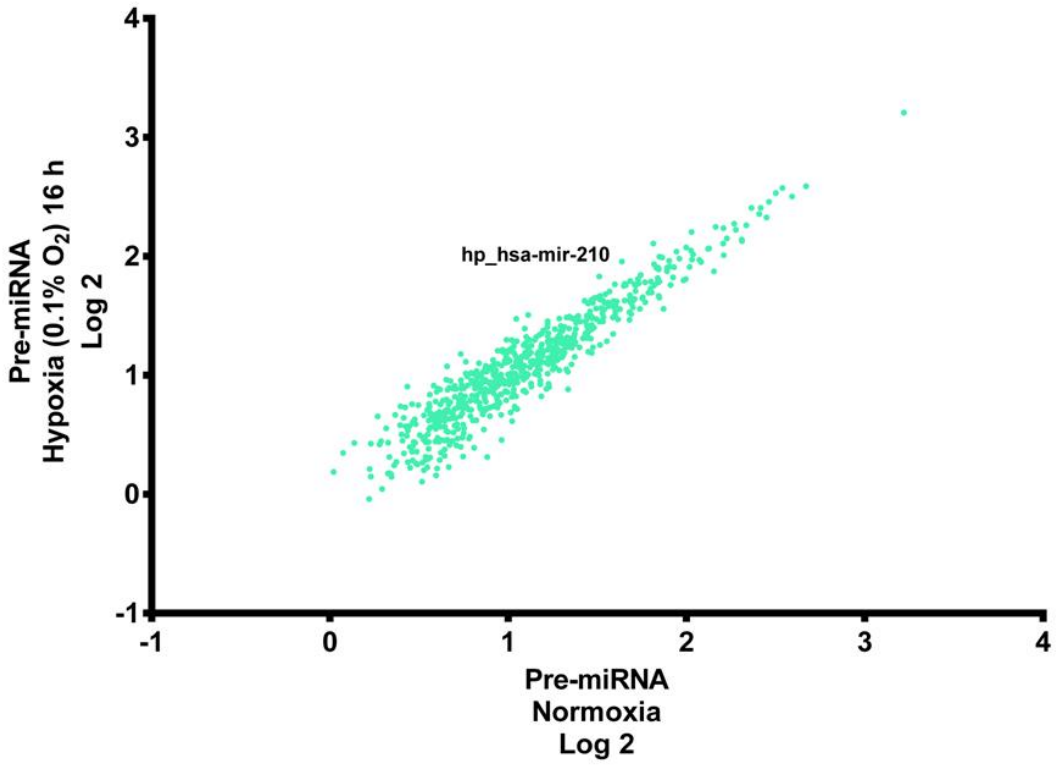

Figure 6.3 Precursor miRNA expression in hypoxia (0.1% O_2 16 h) vs. normoxia.

Microarray analysis. Scatter plot of average probe intensities of precursor miRNAs expressed in MCF7 cells after exposure to hypoxia (0.1% O_2) 16 h vs. normoxia (n=3).

To determine the influence of hypoxia on the processing of each hairpin to a mature miRNA, the ratios between precursor miRNA/mature miRNAs were examined. If hypoxia affected the processing of pre-miRNAs into mature miRNAs, an accumulation of pre-miRNAs was expected. However, no significant changes in ratios were apparent between the ratio pre-miRNA/mature miRNA in hypoxia when compared to normoxia (Figure 6.4). In keeping with the modest changes in DICER expression at this time point, no significant changes were observed in mature or precursor miRNA levels in MCF7 cells exposed to hypoxia (0.1% O_2), compared to normoxia after 16 h.

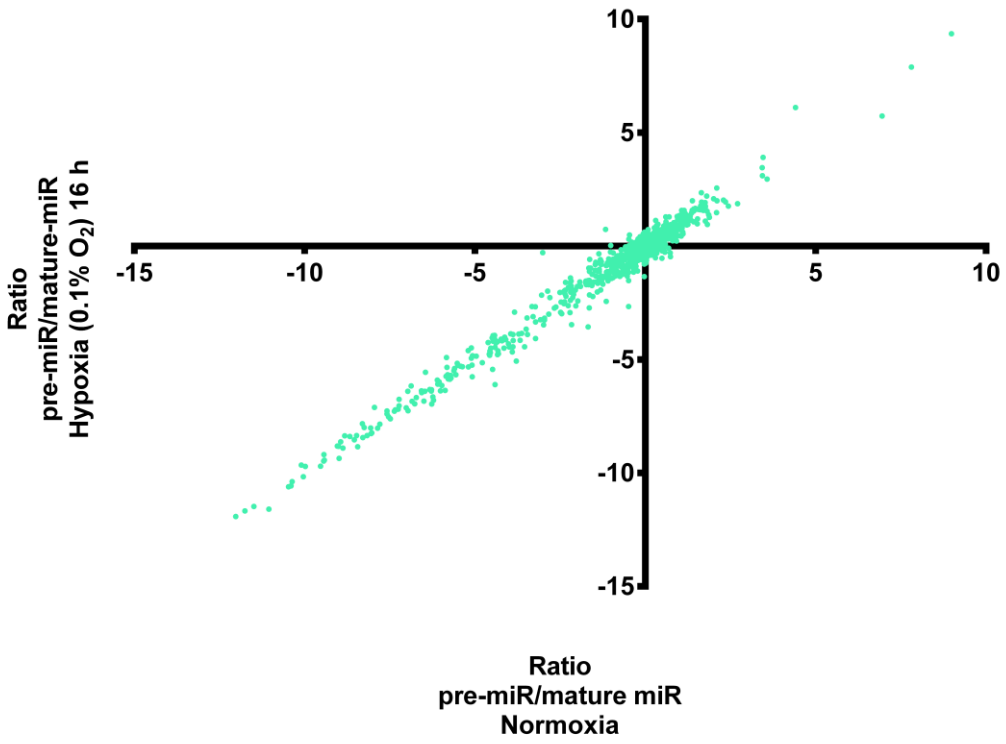

Figure 6.4 Ratios between precursor/mature miRNA expression in hypoxia (0.1% O_2 16 h) vs. normoxia.

Microarray analysis. Scatter plot of average ratios between precursor/mature miRNAs expressed in MCF7 cells after exposure to hypoxia (0.1% O_2) 16 h vs. normoxia (n=3).

A Principle component analysis (PCA) plot (Figure 6.5) was generated using Partek Express software package, to visualise the distribution of expression data within sample sets. Each point represents expression data from a sample microarray and the positions of each point are relative to each other. Arrays with similar gene expression cluster together. Here the three replicates of each treatment (hypoxia-red dots and normoxia-blue dots) did not cluster together as expected indicating that overall there was considerable variation in mature and precursor miRNA expression between the three replicates of hypoxia-treated samples or normoxia-treated samples. Also the variability within treatments ensured that only limited separation was observed between the hypoxic and normoxic array datasets.

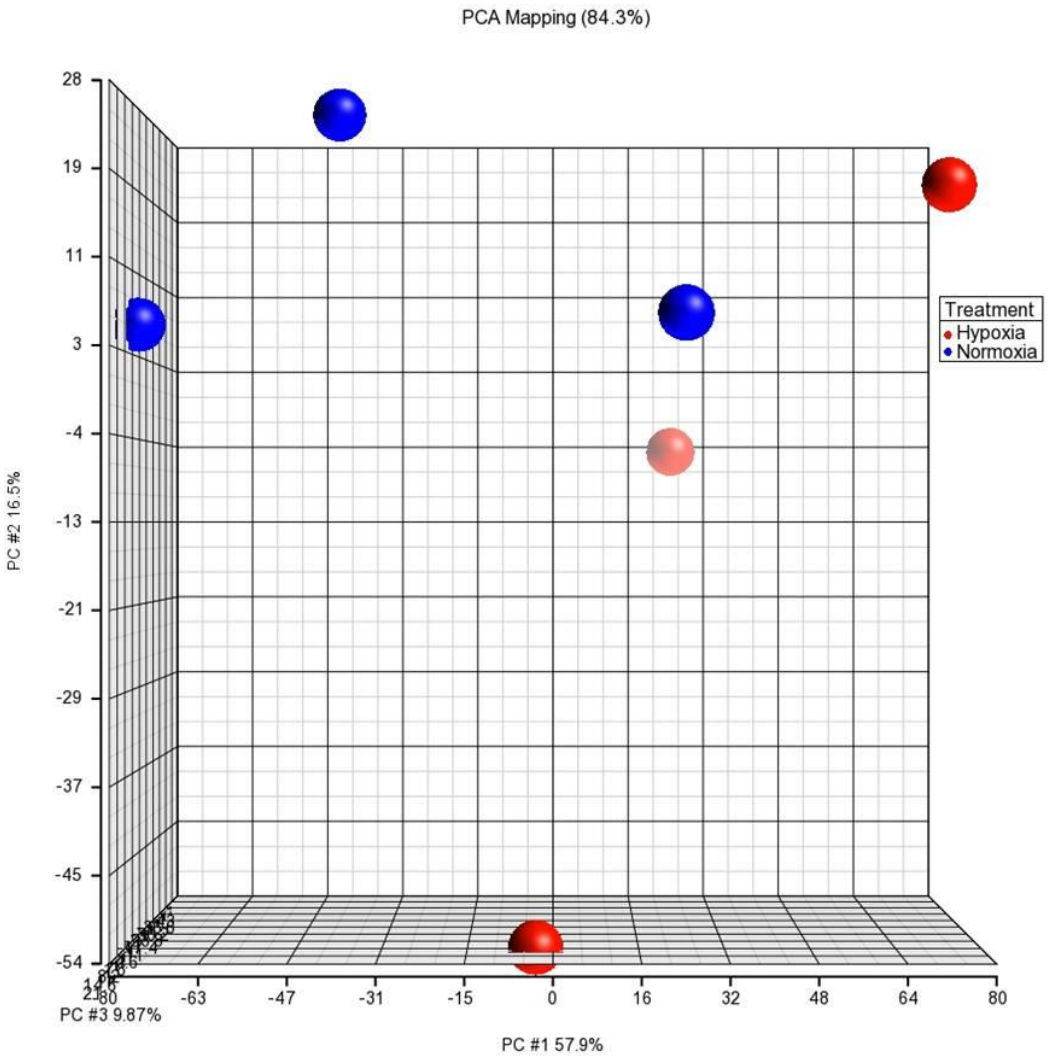

Figure 6.5 Principle component analysis plot of microRNA expression values from MCF7 cells in hypoxia (0.1% O_2 16 h) vs. normoxia.

Mature and precursor miRNA expression in MCF7 cells after exposure to hypoxia (0.1% O_2) 16 h or normoxia. Red dots represent the expression values of samples exposed to hypoxia and blue dots represent the expression values of samples exposed to normoxia.

6.3.1.2 Microarray analysis after exposure to hypoxia for 48 h

As described in previous chapters, significant down regulations of DICER and other miRNA biogenesis proteins were observed only at longer durations (48 h) of hypoxic exposure. Therefore, a similar microrarray experiment was undertaken after exposing MCF7 cells to hypoxia (0.1% O_2) for 48 h. MCF7 cells were exposed to hypoxia or normoxia for 48 h and total RNA from these samples was analysed using the Affymetrix Genechip miRNA Array (version 3.1) system following the same analysis methods as above. There were several miRNAs that were significantly up or down regulated in MCF7 cells after exposure to hypoxia (0.1% O_2) for 48 h when compared to normoxia (Figure 6.6).

Eight miRNAs were significantly up regulated (Table 6.3), and four miRNAs were significantly down regulated in hypoxia when compared to normoxia (Table 6.4). Of the eight up regulated miRNAs, miR-210 has been well validated as a highly induced miRNA in hypoxia (Camps et al., 2008). For the other hypoxically regulated miRNAs interesting links to tumour pathogenesis have been reported. Previous work reported miR-193 to be a tumour suppressor and expressed at significantly low levels in malignant melanoma (Chen et al., 2011). miR-193 can repress myeloid cell leukaemia sequence 1 in melanoma cells (Chen et al., 2011). miR-1972 was identified as a novel miRNA in acute lymphoblastic leukaemia cells (Schotte et al., 2009). miR-181a has roles in vascular development (Kazenwadel et al., 2010), and cancer progression (Fei et al., 2012, Ke et al., 2012). Previous work showed that miR-181a was induced in gastric cancers and was involved in repressing tumour suppressor KLF6 (Kruppel like factor 6) (Zhang et al., 2012). Ke et al. (2012) reported higher expression of miR-181a was observed in human cervical cancer specimens and cell lines that were insensitive to radiation therapy (Ke et al., 2012).

Expression levels of miR-23a and miR-27a are deregulated in many cancers (Li et al., 2013, Wang et al., 2012). miR-23a was reported to promote colorectal cancer progression (Wang et al., 2012). miR-23a and miR-27a both have been found to promote cell invasion and metastasis both in breast and hepatic cancers (Li et al., 2013). Interestingly hypoxic regulation of the passenger strand (or star form) of several miRNAs was seen (miR-23a-5p, miR-27a-5p, miR-181a-3p and miR-193b-5p) though the functional consequences of such regulation is unclear.

A miRNA down regulated in hypoxia, miR-205 regulates epithelial to mesenchymal transistion by targeting ZEB1 and SIP1 genes in tumour metastasis (Gregory et al., 2008). miR-149 was also significantly down regulated in hypoxia and previous reports showed reduced expression of miR-149 in colorectal cancer tissue when compared to non-cancerous tissue (Wang et al., 2013). miR-33b-3p another miRNA that was significantly down regulated in hypoxia showed a 5.12 fold change in hypoxia compared to normoxia. Previous work showed both strands of this miRNA miR-33b/miR-33b-3p were involved in regulating lipid metabolism (Goedeke et al., 2013). Significant up regulation with a large fold change was observed for miR-3193 in hypoxia compared to normoxia. miR-3193 is a recently identified miRNA and there is no current validated information about genes regulated by this miRNA

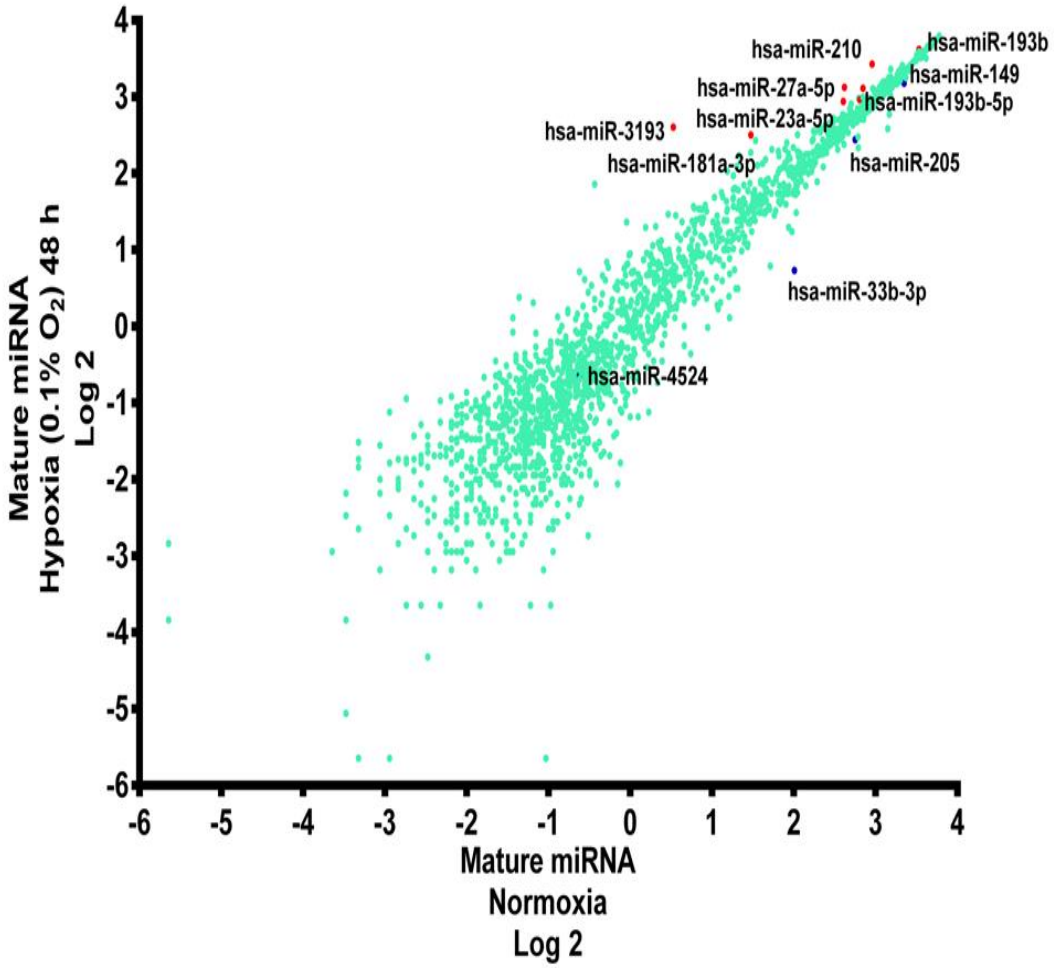

Figure 6.6 Mature miRNA expression in hypoxia (0.1% O_2 48 h) vs. normoxia.

Microarray analysis. Scatter plot of average probe intensities of mature miRNAs expressed in MCF7 cells after exposure to hypoxia (0.1% O_2) 48 h vs. normoxia (n=3). ● indicates mature miRNAs that were significantly up regulated in hypoxia. ● indicates mature miRNAs that were significantly down regulated in hypoxia. Differentially expressed genes were determined using ANOVA and P value adjusted with FDR (False discovery rate) analysis. $P<0.05$ was considered as a significant change.

Table 6.3 miRNAs up regulated in MCF7 cells after exposure to hypoxia (0.1% O_2) vs. normoxia for 48 h.

miRNA	P value	Fold change
hsa-miR-3193	0.0349	24.85
hsa-miR-210	0.0007	8.05
hsa-miR-181a-3p	0.0418	7.33
hsa-miR-27a-5p	0.0104	5.93
hsa-miR-23a-5p	0.0413	3.01
hsa-miR-193b-5p	0.0104	2.76
hsa-miR-1972	0.0309	1.71
hsa-miR-193b	0.0104	1.68

Table 6.4 miRNAs down regulated in MCF7 cells after exposure to hypoxia (0.1% O_2) vs. normoxia for 48 h.

miRNA	P value	Fold change
hsa-miR-4521	0.0008	-7.35
hsa-miR-33b-3p	0.0360	-5.12
hsa-miR-205	0.0307	-2.47
hsa-miR-149	0.0077	-2.19

As in the previous array the hairpin (hp) intensity values included both primary and precursor miRNAs. Similar to the 16 h hypoxic exposure, there were no significant changes in precursor miRNA levels in hypoxia (0.1% O_2), compared to normoxia at 48 h (Figure 6.7). Even though not significant, the expression of hp-miR-210 was induced in hypoxia (0.1% O_2), compared to normoxia at 48 h.

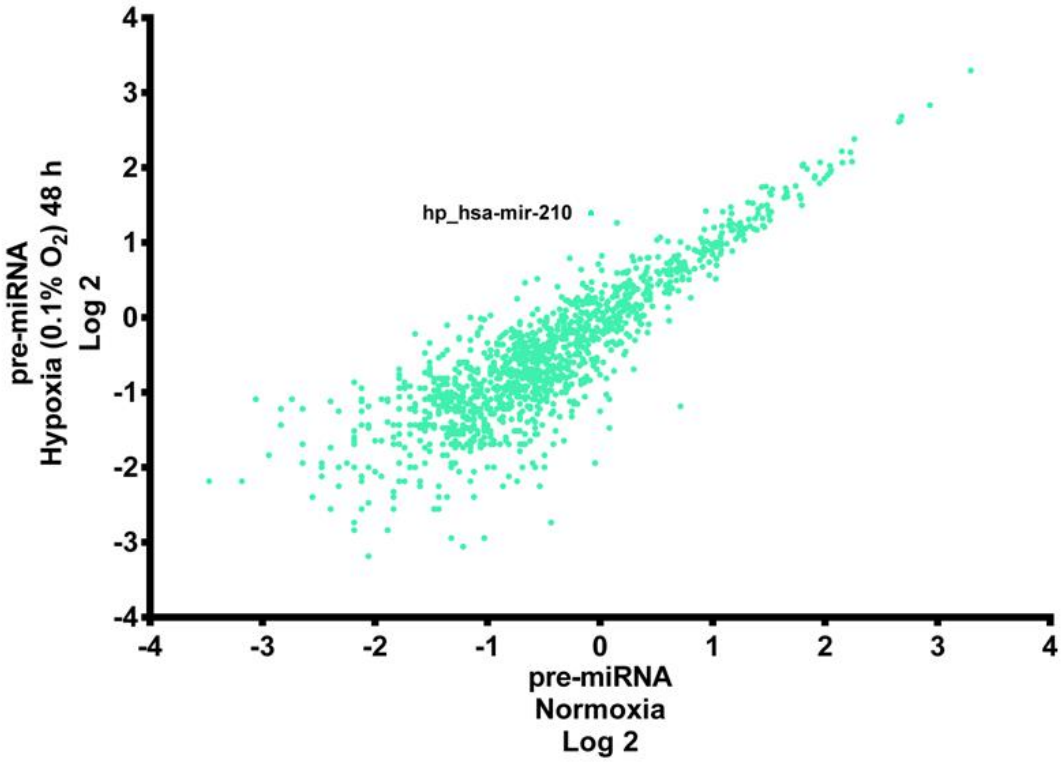

Figure 6.7 Precursor miRNA expression in hypoxia (0.1% O_2 48 h) vs. normoxia.

Microarray analysis. Scatter plot of average probe intensities of precursor miRNAs expressed in MCF7 cells after exposure to hypoxia (0.1% O_2) 48 h vs. normoxia (n=3).

To determine the influences of hypoxia on processing of each hairpin to a mature miRNA in hypoxia compared to normoxia, the ratios between precursor/mature miRNAs were examined. Even after a longer duration of hypoxic exposure (0.1% O_2 for 48 h) there were no significant changes in precursor/mature miRNA ratios (Figure 6.8). The overall mean ratios between pre-miRNA/mature miRNA were not significantly different between hypoxia (1.36) and normoxia (1.30).

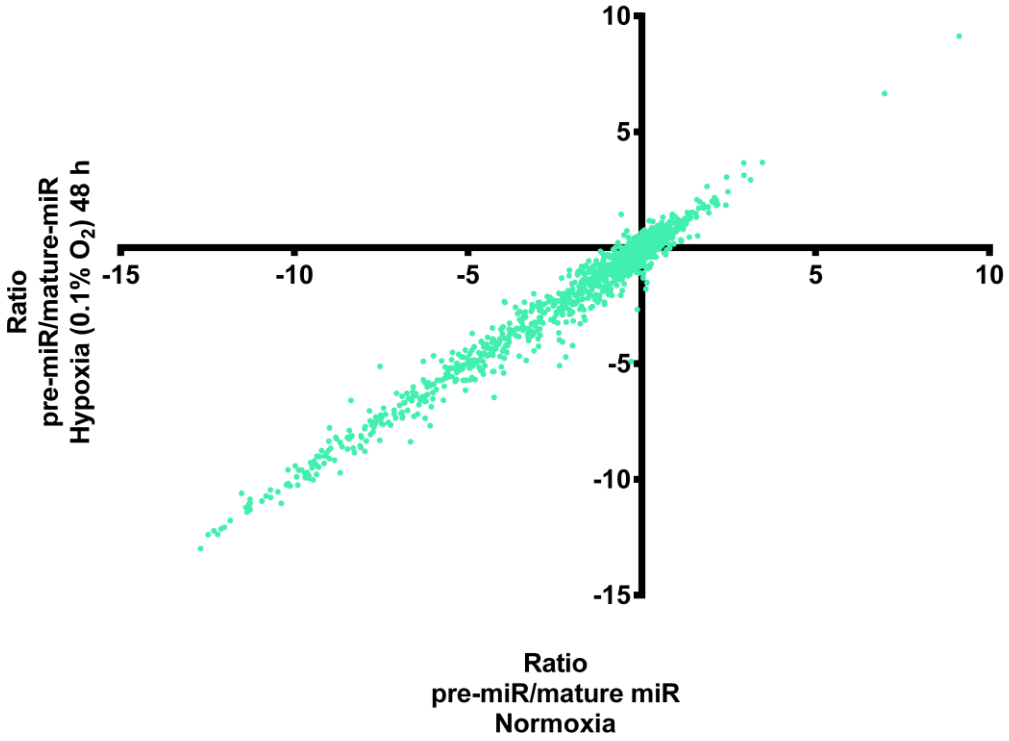

Figure 6.8 Ratios between precursor/mature miRNA expression in hypoxia (0.1% O_2 48 h) vs. normoxia.

Microarray analysis. Scatter plot of average ratios between precursor/mature miRNAs expressed in MCF7 cells after exposure to hypoxia (0.1% O_2) 48 h vs. normoxia (n=3).

6.3.1.3 Microarray analysis after DICER inhibition

In addition to the RNA from hypoxic and normoxic cells, three replicates with DICER RNA interference were also included in the second microarray experiment, with the intention of identifying and comparing effects of *DICER* inhibition on mature and precursor miRNA levels. DICER expression was inhibited in MCF7 cells using transient transfection of siRNAs targeting *DICER*. A double transfection was performed following the same protocol as before (as described in chapter 2). After 24 h following the first transfection a second transfection was performed using exactly the same method. A significant inhibition of DICER protein levels was observed after the double transfections using siRNAs targeting *DICER* in MCF7 cells (Figure 6.9). RNA was extracted using the Trizol protocol and RNA from these samples was analysed using the Affymetrix Genechip miRNA Array (version 3.1) system. Mature and precursor miRNA expression levels between *DICER* inhibition, hypoxia and normoxia were compared. Intensity data were normalised using the quantile normalisation method and $\log_2$ transformed. Gene expression data was analysed using the Partek Express software package. Differentially expressed genes were determined using ANOVA, and P values were adjusted with FDR (False discovery rate) analysis. $P<0.05$ was considered as a significant change.

Some mature miRNAs were up or down regulated after *DICER* RNA interference in MCF7 cells (Figure 6.9). Forty five miRNAs were significantly up regulated (Table 6.5) after DICER inhibition, while nineteen miRNAs (Table 6.6) were significantly down regulated after DICER inhibition in MCF7 cells. The increase in mature miRNA levels after DICER inhibition was an unexpected result, as mature miRNA levels were expected to be down regulated with DICER inhibition due to a reduction in miRNA processing.

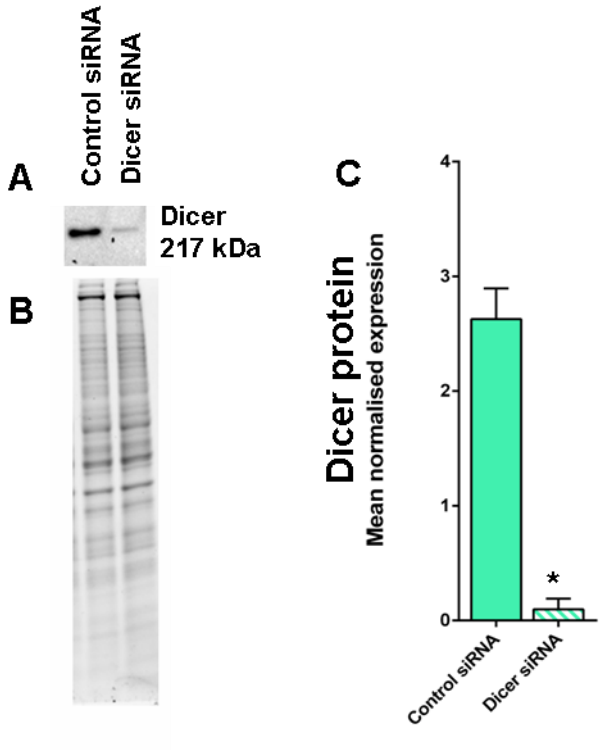

Figure 6.9 DICER protein levels in MCF7 cells after transfections with siRNAs targeting DICER.

Protein levels were examined by immunoblotting. A, DICER protein levels in MCF7 cells after double transient transfections with siRNAs targeting *DICER* or control siRNAs (P=0.002). B, Stain-free image of SKBR3 cell lysates separated on TGX Stain-Free 'Any kD' SDS gel, transferred onto the PVDF membrane. C, Densitometric analysis following normalisation with total protein on PVDF membrane. Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $P < 0.05$ compared with parallel controls.

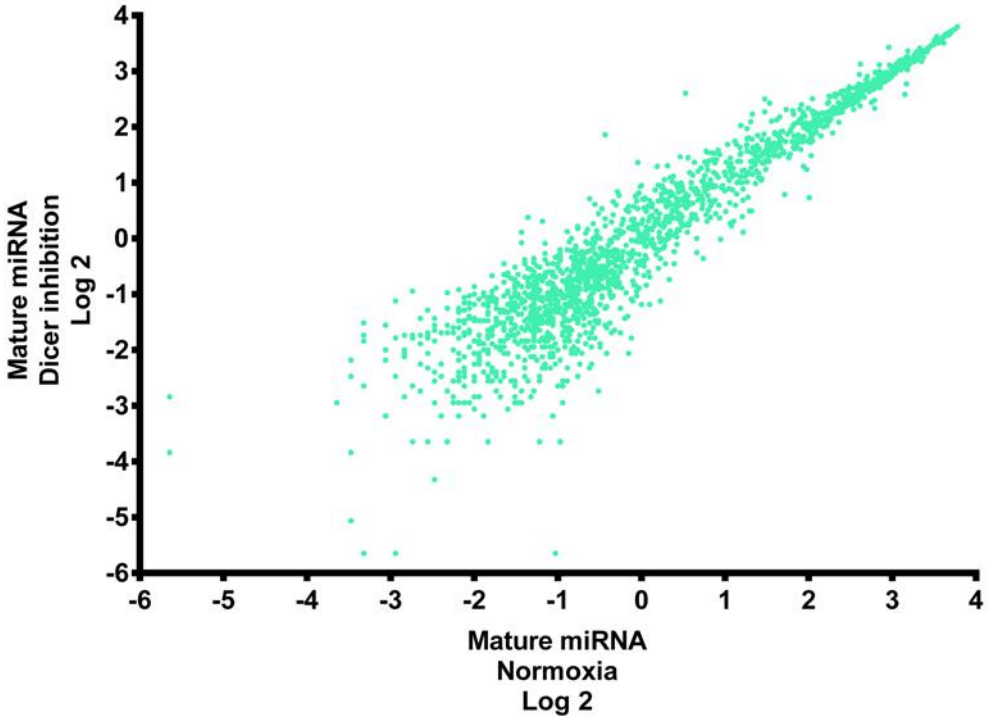

Figure 6.10 Mature miRNA expression after DICER inhibition vs. normoxia.

Microarray analysis. Scatter plot of average probe intensities of mature miRNAs expressed in MCF7 cells after DICER inhibition using siRNAs vs. normoxia (n=3).

Table 6.5 miRNAs up regulated in MCF7 cells after DICER inhibition vs. normoxia.

miRNA	P value	Fold-Change
hsa-miR-1231_st	0.0406	3.16
hsa-miR-4734_st	0.0144	3.11
hsa-miR-1908_st	0.0277	3.00
hsa-miR-3185_st	0.0088	2.94
hsa-miR-4741_st	0.0163	2.88
hsa-miR-4521_st	0.0088	2.83
hsa-miR-3196_st	0.0226	2.83
hsa-miR-1228-5p_st	0.0198	2.77
hsa-miR-762_st	0.0088	2.74
hsa-miR-3940-5p_st	0.0144	2.71
hsa-miR-3937_st	0.0271	2.71
hsa-miR-4532_st	0.0145	2.67
hsa-miR-3178_st	0.0088	2.66
hsa-miR-4707-5p_st	0.0146	2.66
hsa-miR-4651_st	0.0198	2.65
hsa-miR-27a-5p_st	0.0301	2.64
hsa-miR-663_st	0.0220	2.63
hsa-miR-4745-5p_st	0.0088	2.57
hsa-miR-4488_st	0.0249	2.54
hsa-miR-4695-5p_st	0.0267	2.49
hsa-miR-4505_st	0.0267	2.43
hsa-miR-638_st	0.0146	2.40
hsa-miR-4800-5p_st	0.0406	2.40
hsa-miR-149-star_st	0.0283	2.39
hsa-miR-4466_st	0.0197	2.38
hsa-miR-4516_st	0.0211	2.33
hsa-miR-2861_st	0.0255	2.30
hsa-miR-4787-5p_st	0.0144	2.29
hsa-miR-3656_st	0.0144	2.26
hsa-miR-4763-3p_st	0.0434	2.22
hsa-miR-1469_st	0.0146	2.21
hsa-miR-1915_st	0.0163	2.16

hsa-miR-3960_st	0.0144	2.11
hsa-miR-4484_st	0.0432	2.11
hsa-miR-4417_st	0.0144	2.08
hsa-miR-4433_st	0.0404	2.03
hsa-miR-3665_st	0.0149	1.95
hsa-miR-1972_st	0.0144	1.93
hsa-miR-4497_st	0.0146	1.89
hsa-miR-25-5p_st	0.0464	1.80
hsa-miR-4687-3p_st	0.0255	1.79
hsa-miR-4689_st	0.0406	1.79
hsa-miR-4463_st	0.0424	1.72
hsa-miR-1268b_st	0.0201	1.66
hsa-miR-629-5p_st	0.0454	1.57

Table 6.6 Mature miRNAs down regulated in MCF7 cells after DICER inhibition vs. normoxia.

miRNA	**P value**	**Fold-Change**
hsa-miR-769-3p_st	0.0144	-3.22
hsa-miR-769-5p_st	0.0424	-2.67
hsa-miR-3200-3p_st	0.0386	-2.59
hsa-miR-501-5p_st	0.0088	-2.55
hsa-miR-99b-3p_st	0.0146	-2.45
hsa-miR-675_st	0.0418	-2.19
hsa-miR-421_st	0.0038	-2.15
hsa-miR-589-39_st	0.0249	-2.10
hsa-miR-324-5p_st	0.0198	-2.05
hsa-miR-205_st	0.0268	-2.04
hsa-miR-330-3p_st	0.0310	-2.04
hsa-miR-671-5p_st	0.0198	-1.90
hsa-miR-149_st	0.0111	-1.84
hsa-miR-301a_st	0.0249	-1.73
hsa-miR-744_st	0.0327	-1.70
hsa-miR-500a_st	0.0406	-1.67
hsa-miR-1301_st	0.0268	-1.62
hsa-miR-99b_st	0.0197	-1.59

hsa-miR-423-3p_st	0.0144	-1.56

Few pre-miRNAs were up or down regulated after DICER inhibition in MCF7 cells (Figure 6.10). Eight pre-miRNAs were significantly up regulated after DICER inhibition in MCF7 cells (Table 6.7). Out of these, three of the corresponding mature miRNAs were also significantly up regulated after DICER inhibition in MCF7 cells (miR- 3185, miR-3960 and miR-4516). Two pre-miRNAs were significantly down regulated after DICER inhibition in MCF7 cells (Table 6.8).

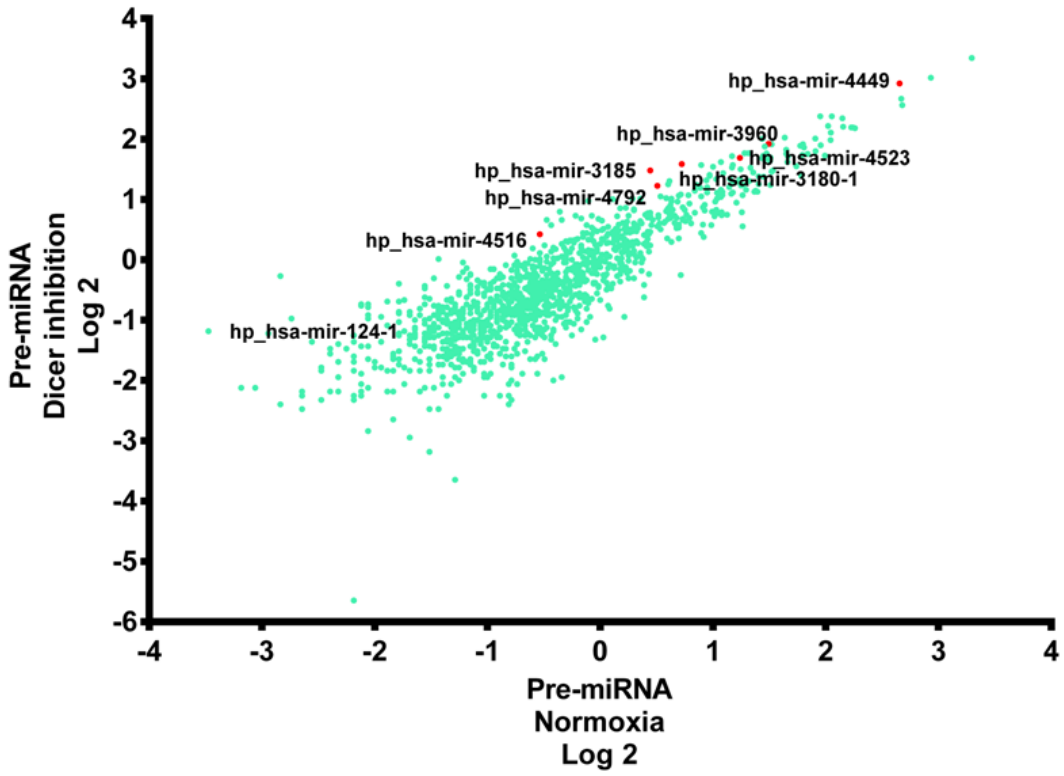

Figure 6.11 Precursor miRNA expression after DICER inhibition vs. normoxia.

Scatter plot of average probe intensities of precursor miRNAs expressed in MCF7 cells after DICER inhibition vs. normoxia (n=3). * indicates hairpins that showed a significant increase after DICER inhibition.

Table 6.7 Pre-miRNAs up regulated in MCF7 cells after DICER inhibition.

Pre-miRNA	P value	Fold-Change
hp_hsa-mir-3185	0.0468	2.70
hp_hsa-mir-3180-1	0.0136	2.57
hp_hsa-mir-124-3	0.0406	2.49
hp_hsa-mir-4449	0.0432	2.46
hp_hsa-mir-3960	0.0468	1.95
hp_hsa-mir-4792_st	0.0088	1.92
hp_hsa-mir-4523_st	0.0440	1.82
hp_hsa-mir-4516	0.0379	1.58

Table 6.8 Pre-miRNAs down regulated in MCF7 cells after DICER inhibition.

Pre-miRNA	P value	Fold-Change
hp_hsa-mir-99b_st	0.0144	-1.77
hp_hsa-mir-324_st	0.0365	-1.58

When the ratios between pre-miRNA/mature miRNA were calculated surprisingly there were no significant changes following *DICER* inhibition (Figure 6.11). The overall mean ratios between pre-miRNA/mature miRNA were not significantly different between *DICER* inhibition (1.33) or normoxia (1.30).

No significant changes in mature/precursor miRNA ratios were seen either with longer exposure of hypoxia (48 h) (Figure 6.9) or surprisingly following DICER suppression by RNA interference (Figure 6.11).

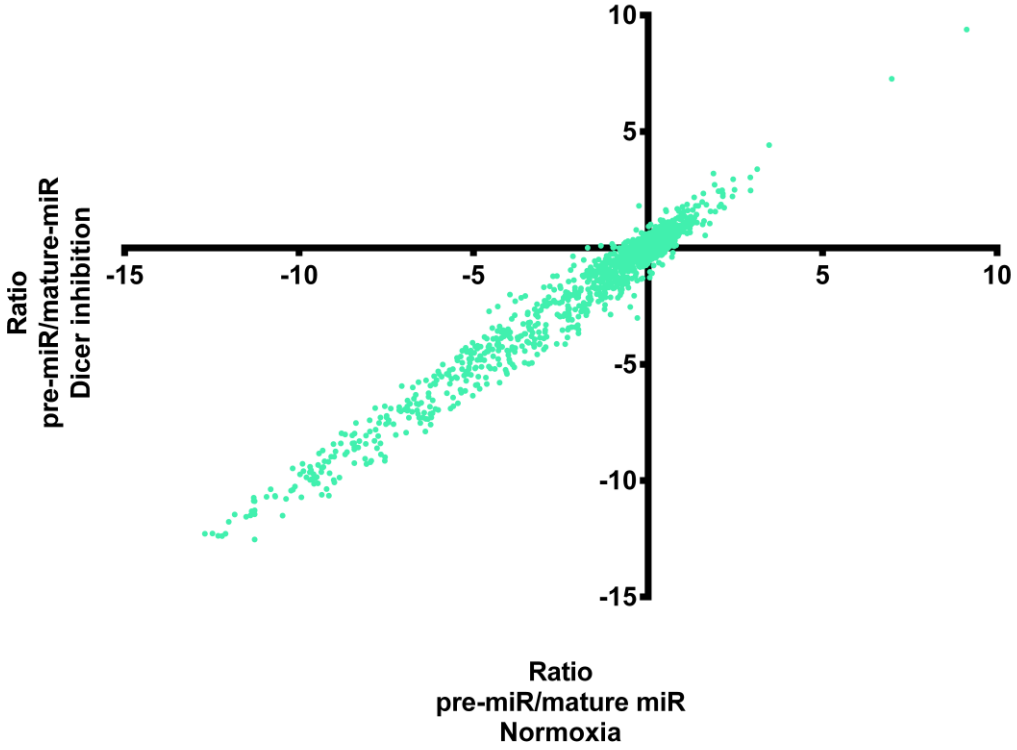

Figure 6.12 Ratio between precursor/mature miRNA expression after DICER inhibition vs. normoxia.

Scatter plot of average ratios between precursor/mature miRNAs expressed in MCF7 cells after DICER inhibition vs. normoxia (n=3).

A PCA plot (Figure 6.13) was generated using Partek Express software package, to visualise the distribution of expression data within sample sets. Each point represents expression data from a sample microarray and the positions of each point are relative to each other. Arrays with similar gene expression cluster together. Here the three replicates of each treatment (hypoxia-blue dots, normoxia-green dots and DICER inhibition-red dots) did group together, and there was separation between the three different groups. This indicates that overall there was some variation in mature and precursor miRNA expression between the three treatments; hypoxia-treated samples, normoxia-treated samples or *DICER* inhibited samples.

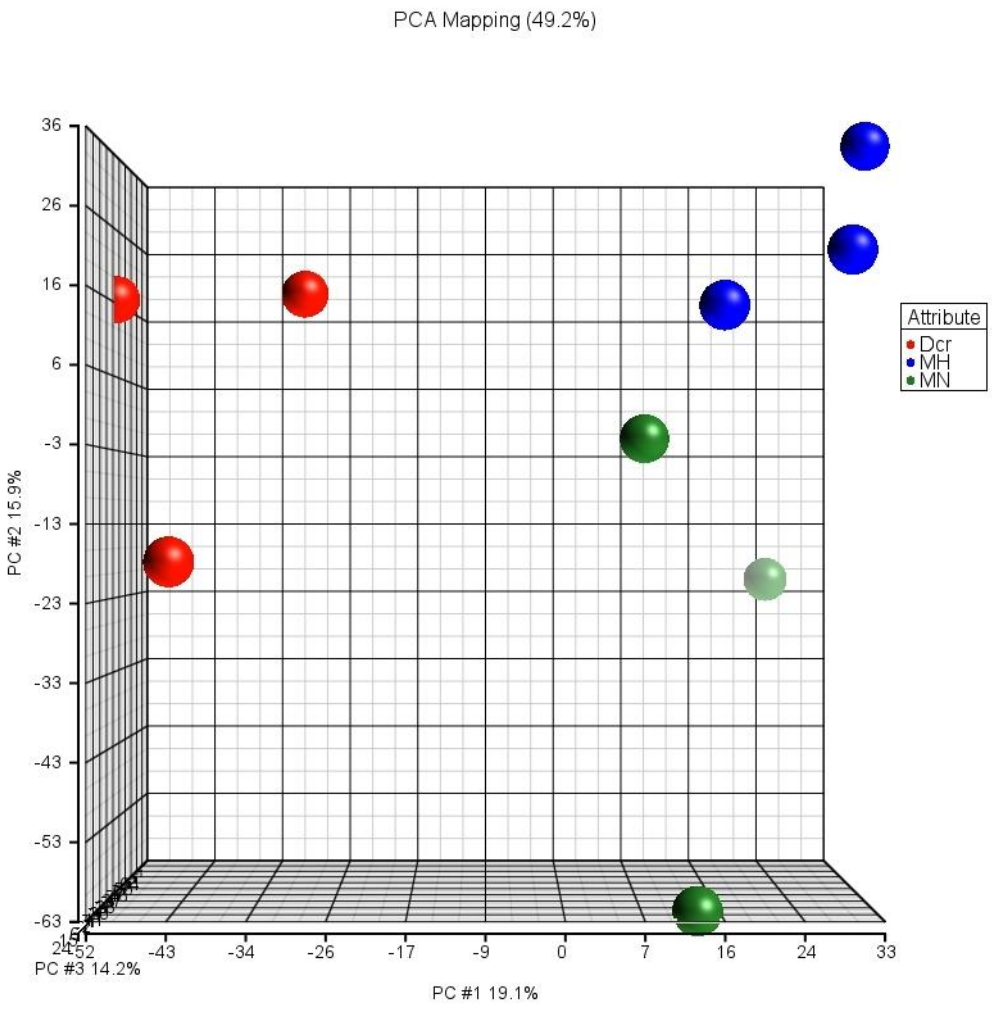

Figure 6.13 Principle component analysis plot of microRNA expression values from MCF7 cells in hypoxia (0.1% O_2) 48 h, normoxia and *DICER* inhibition.

Mature and precursor miRNA expression in MCF7 cells after exposure to hypoxia (0.1% O_2) 48 h, normoxia or after *DICER* inhibition. Blue dots represent the expression values of samples exposed to hypoxia, green blue dots represent the expression values of samples exposed to normoxia, and red dots represent the expression values of samples after DICER inhibition.

6.3.2 Mature and precursor miRNA expression analysis by real time RT-PCR

To explore the effects of hypoxia on the processing of miRNAs with an independent assay, the mature and precursor miRNA levels of let-7a, miR-21 and miR-185 in MCF7 cells exposed to hypoxia or normoxia were measured by real time RT-PCR. For the parallel detection of mature and pre-miRNA levels, cDNA was prepared using the Qiagen miScript II RT kit. Mature miRNA levels were assayed using the specific miScript primer assays and precursor miRNAs were assayed using miScript precursor assays.

The expression levels of a few selected mature and corresponding precursor miRNAs were examined in MCF7 cells exposed to hypoxia (0.1% O_2) compared to normoxia for 48 h. These miRNAs (let-7a, miR-21 and miR-185) were chosen as previous reports showed they were DICER dependent miRNAs, and *DICER* RNA interference reduced mature miRNA expression (Bu et al., 2009, Suarez et al., 2007, Ho et al., 2012). Suarez et al. (2007) looked at let-7a and miR-16 levels in EA.hy.926 cells 60 h after transfecting with *DICER* specific siRNA or control siRNA. They saw significant down regulation of let-7a and miR-16 levels after *DICER* inhibition (Suarez et al., 2007). Another study showed significant reduction in miR-21 expression in MCF7 cells after *DICER* siRNA treatment (Bu et al., 2009). A recent study by Ho et al. (2012) reported miR-185 as a DICER dependent miRNA and showed significant down regulation of mature miR-185 expression and also accumulation of the pre-miR-185 after DICER inhibition in HUVECs (Ho et al., 2012).

Only a modest decrease in mature and precursor levels of let-7a and miR-21 in hypoxia was observed. There was no accumulation of pre-let-7a or pre-miR-21 in hypoxia (Figure 6.14 and Figure 6.15). A significant reduction (P=0.03) in mature miR-185 was observed in hypoxia in MCF7 cells (Figure 6.16), but no accumulation of precursors were seen.

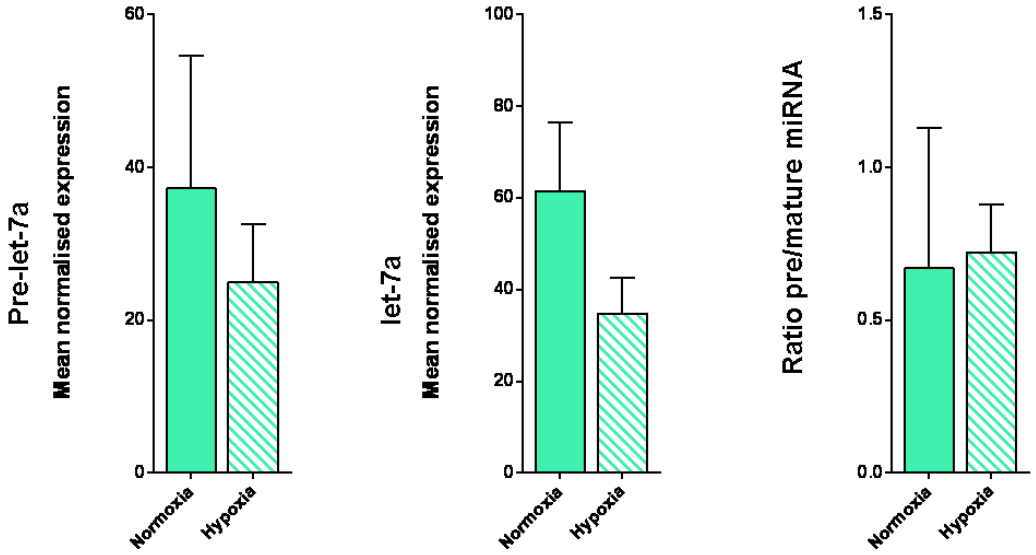

Figure 6.14 Mature and precursor let-7a expression in hypoxia vs. normoxia.

miRNA levels were analysed by real time RT-PCR and normalised to RNU6B nucleolar RNA levels. Pre-let-7a and mature let-7a expression, precursor/mature ratio in MCF7 cells exposed to hypoxia (0.1% O_2) 48 h vs. normoxia. Data represent normalized mean ±S.E (error bars) (n=3).

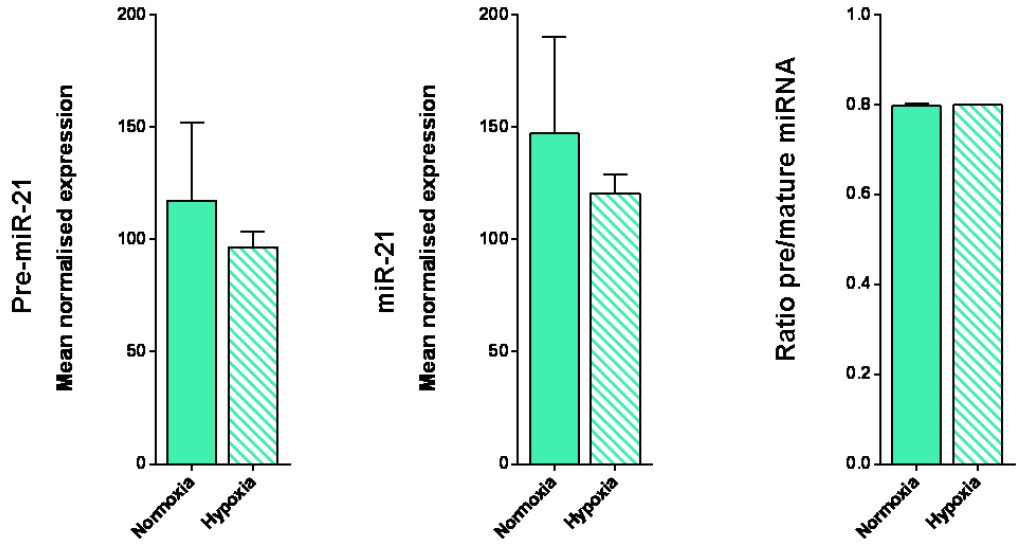

Figure 6.15 Mature and precursor miR-21 expression in hypoxia vs. normoxia.

miRNA levels were analysed by real time RT-PCR and normalised to RNU6B nucleolar RNA levels. Pre-miR-21 and mature miR-21 expression, precursor/mature ratio in MCF7 cells exposed to hypoxia (0.1% O_2) 48 h vs. normoxia. Data represent normalized mean ±S.E (error bars) (n=3).

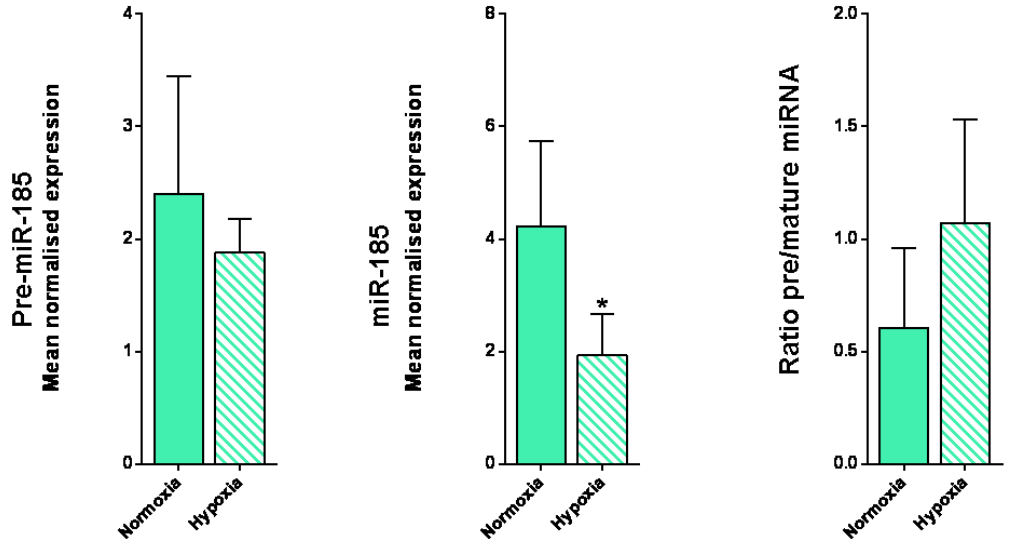

Figure 6.16 Mature and precursor miR-185 expression in hypoxia vs. normoxia.

miRNA levels were analysed by real time RT-PCR and normalised to RNU6B nucleolar RNA levels. Pre-miR-185 and mature miR-185 (p=0.03) expression, precursor/mature ratio in MCF7 cells exposed to hypoxia (0.1% O_2) 48 h vs. normoxia. Data represent normalized mean ±S.E (error bars) (n=3). Statistical significance tested for with student's t-test. * denotes $P < 0.05$ compared with parallel controls.

6.4 MicroRNA mediated gene suppression is affected by hypoxia

After seeing the multiple potential influences of hypoxia on miRNA biogenesis proteins, the effect of hypoxia on miRNA mediated gene suppression was examined. For this experiment a plasmid containing the 3'UTR of the ZEB1 gene downstream of a Renilla luciferase (RL) reporter (Gregory et al., 2008) (Figure-6.15A) and a plasmid expressing a pre-miR-200b was used (Figure 6.15C). ZEB1 is an E-cadherin transcriptional repressor involved in the epithelial to mesenchymal transition of tissues and is regulated by the miR-200 family (Gregory et al., 2008). ZEB1 gene has miR-200b target sites and binding of the mature miR-200b will repress the RL activity.

SKBR3 cells were co-transfected with a plasmid containing the 3'UTR from the ZEB1 gene downstream of a RL reporter (pCi-neo-ZEB1-hRL) (Figure-6.15A) together with the precursor miR-200b expressing (pCMV-miR-200b) (Figure 6.14C) or empty vector (pCMV-miR) (Figure 6.14D), then exposed cells to hypoxia (0.1% O_2 for 24h) or normoxia. PGL3-control plasmid (Figure 6.15B) was used as a control and to normalise the luminescence. Cells were lysed after 24 h and luminescence was measured using a plate reader luminometer.

The luminescence measured with pCi-neo-ZEB1-hRL + pCMV-miR-200b showed a significant repression in normoxia when compared to hypoxia. Two steps are involved in miRNA mediated gene regulation following the artificial introduction of a pre-miRNA. First the pre-miR-200b should be processed to a mature miRNA and then, the mature miR-200b should bind to the miR-200b target sites on the ZEB1 gene. The significant relative repression of luminescence in normoxia suggests less

miR-200b mediated suppression of the ZEB1 gene in hypoxia, compared to normoxia. No change in luminescence was seen in the control (pCi-neo-ZEB1-hRL + pCMV-miR) transfection in hypoxia compared with normoxia (Figure 6.16).

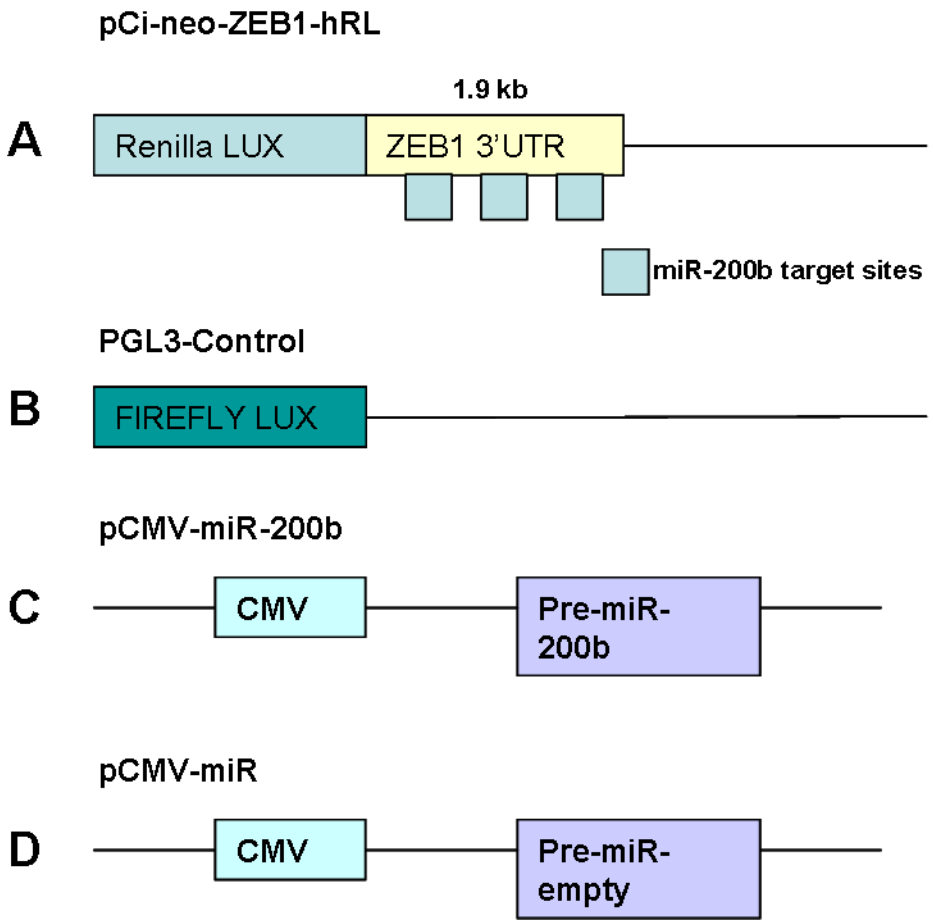

Figure 6.17 Schematic representations of the reporter constructs.

A, pCi-neo-ZEB1-hRL with the 3'UTR from the ZEB1 gene downstream of a Renilla luciferase reporter. B, PGL3 control plasmid with firefly luciferase reporter. C, pCMV-miR-200b pre-miR-200b expression construct. D, pCMV-miR empty vector without the pre-miR-200b insert.

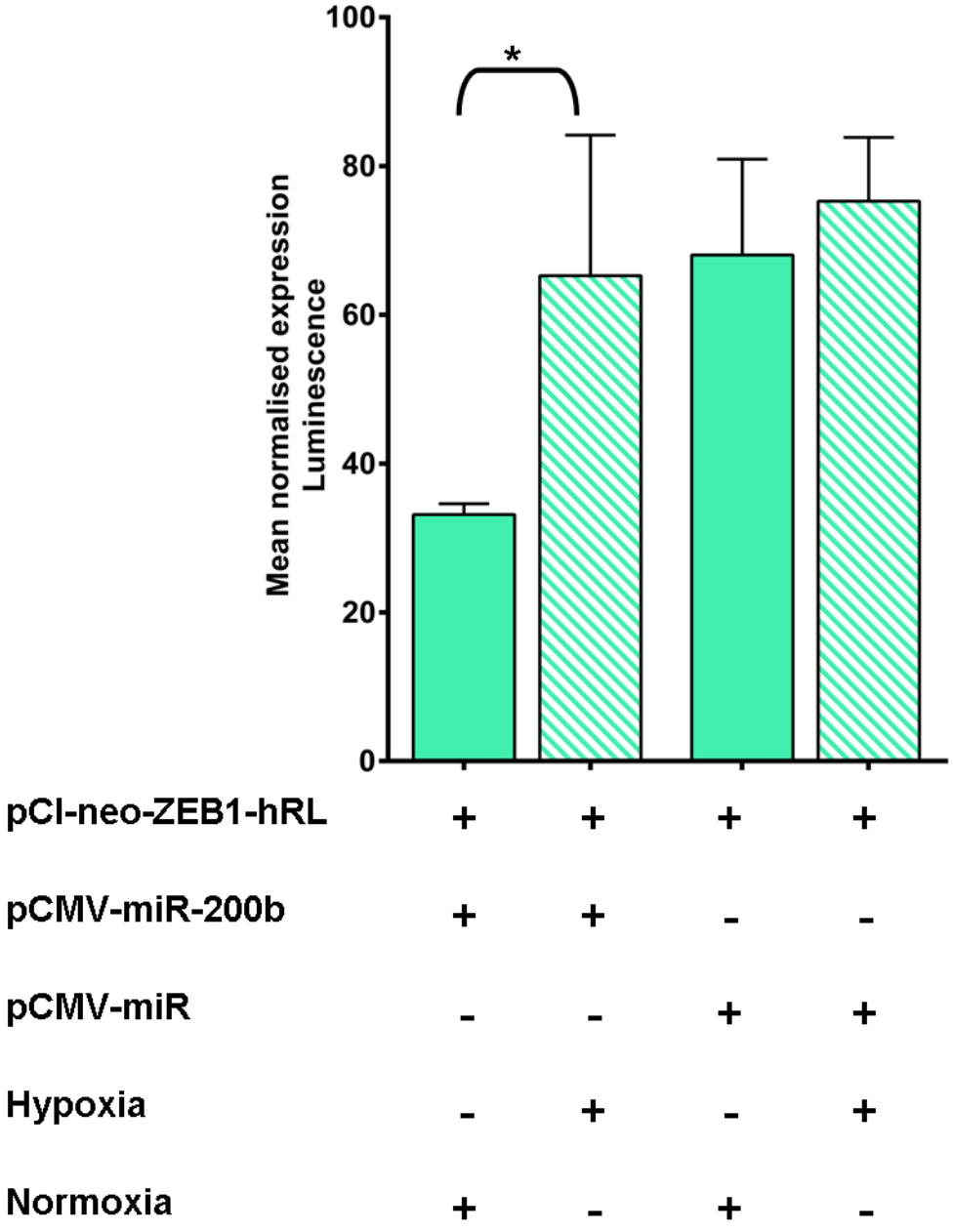

Figure 6.18 miRNA processing is affected by hypoxia.

Luciferase activity in SKBR3 cells after co-transfection with RL reporter containing a ZEB1 WT with miR-200b target sites (pCi-neo-ZEB1-hRL) and precursor miR-200b (pCMV-miR-200b) or empty vector (pCMV-miR) (Origene), then exposure to hypoxia (0.1% O_2 24 h) *vs*. normoxia (P=0.04). Data represent normalized mean ±S.E (error bars) (n=3). * denotes $P < 0.05$ compared with parallel controls.

6.5 Role of DICER in miRNA processing

DICER inhibition by RNAi, or significant down regulation of DICER in hypoxia did not seem to have the significant effect on overall miRNA processing as expected. Therefore a DICER activity assay was carried out to examine if pre-miRNA processing was affected in hypoxia when compared to normoxia. To determine the DICER activity a ^{32}P-UTP labelled human pre-miRNA-145 substrate was incubated for different time periods with extracts of MCF7 cells exposed to hypoxia (0.1% O_2) or normoxia for 48 h. The processing of the pre-miR-145 substrate was examined over a time course (10, 30, 60 and 120 min). The positive control contained the pre-miR-145 substrate and commercially sourced recombinant DICER. The negative control contained the substrate pre-miR-145 and the buffers without any cell extract or enzyme.

Results shown here represent data from three different experiments. At the end of the first time point (10 min) there was no difference in premiR-145 processing between the hypoxic and the normoxic cell lysates (Figure 6.19). At the second time point (30 min) a slight difference was observed in the pre-miR-145 processing. The normoxic cell lysate processed the pre-miR-145 substrate slightly more efficiently than the hypoxic cell lysate. A substantial difference in the processing efficiency of pre-miR-145 was observed at the third time point of 60 min. Pre-miR-145 was processed much more efficiently with the normoxic cell lysates compared to the hypoxic cell lysates at 60 min. At 120 min there was no difference in processing of the pre-miR-145 substrate by the hypoxic or normoxic cell lysates. Overall, the processing of substrate pre-miR-145 was slightly more efficient in the normoxic cell extracts when compared to the hypoxic cell extracts at earlier time points (Figure 6.20). The

positive control, with recombinant DICER, processed the pre-miRNA-145 substrate very efficiently. A change in the pre-miR-145 levels were seen even after the 10 min incubation with recombinant DICER when compared to the negative control at 10 min. The negative control did not show any changes in pre-miR-145 levels even after the 120 min incubation.

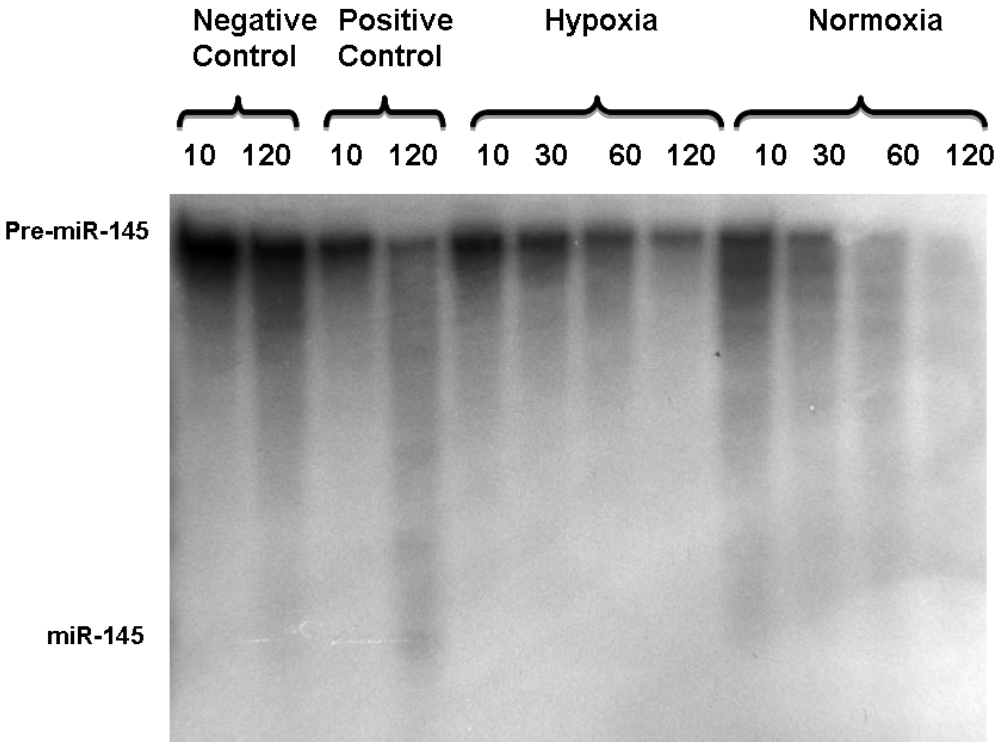

Figure 6.19 RNA gel showing pre-miRNA processing in hypoxia vs. normoxia.

RNA gel showing pre-miR-145 substrate levels after incubating with MCF7 cell lysates exposed to hypoxia (0.1% O_2) 48 h or normoxia over 10, 30, 60 and 120 min. Negative control contained pre-miR-145 substrate with just the buffers. The positive control contained pre-miR-145 substrate with recombinant DICER enzyme.

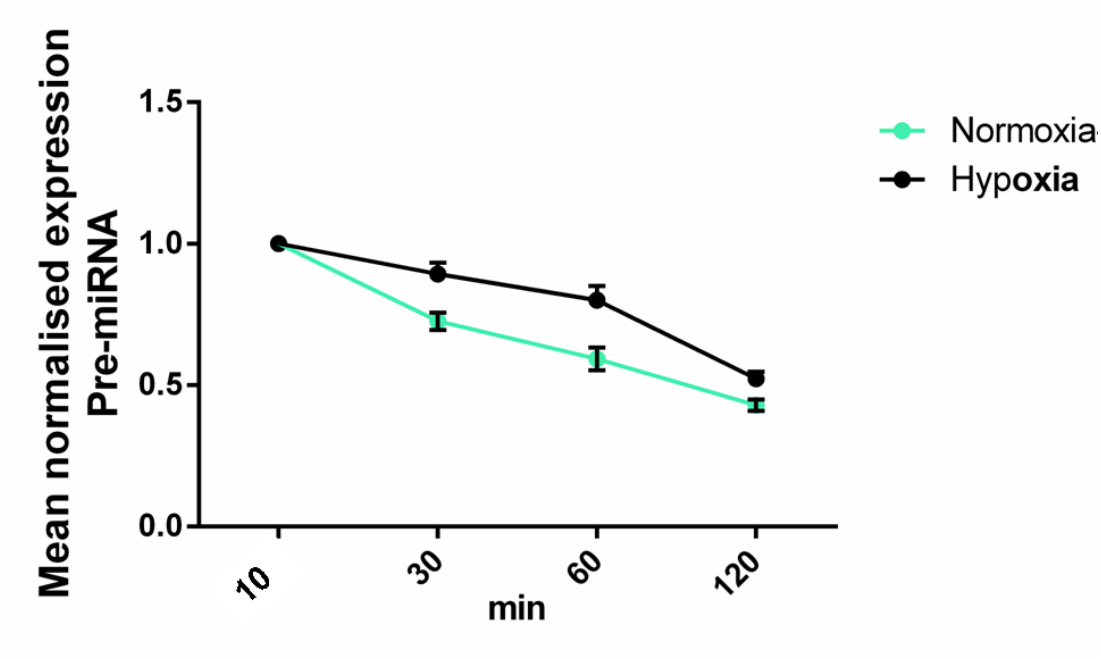

Figure 6.20 Pre-miRNA processing in hypoxia vs. normoxia.

The changes in pre-miR-145 substrate levels over 10, 30, 60 and 120 min after incubation with MCF7 cell lysates exposed to hypoxia (0.1% O_2) 48 h or normoxia. Normalised to the negative control at 10 min. Data represent results from three independent experiments each with three technical replicates.

6.6 Discussion

Previous chapters (3 and 5) of this thesis showed significant and consistent reductions of DICER, TARBP2, DROSHA, DGCR8 and XPO5 proteins after exposure to hypoxia. Even though there was a significant and consistent reduction in the levels of proteins with key roles in miRNA biogenesis under hypoxia, there were no substantial effects on the expression levels of mature miRNAs over the time course of these experiments. Even when DICER levels were suppressed by siRNA only slight alterations in mature miRNA abundance were observed. This was true, both for miRNAs assessed by microarray studies and also when examining the levels of specific miRNAs and their precursors by real time RT-PCR.

Microarray data from MCF7 cells exposed to hypoxia (0.1% O_2) compared with normoxia for 16 h did not show any miRNAs that had a significant fold-change in either direction. After 16 h of hypoxia there was no significant changes in DICER protein levels (as shown in chapter 3), therefore the lack of altered mature miRNA expression in hypoxia compared with normoxia was not surprising. In addition, there were only three replicates per treatment, and this low number of replicates might have also affected the detection of a small significant change. However, surprisingly, microarray data from MCF7 cells exposed to hypoxia (0.1% O_2) vs. normoxia for 48 h also did not show large changes in mature miRNA levels. At 48 h of hypoxia (0.1% O_2), DICER protein levels in MCF7 cells decreased significantly (10 fold), therefore a down regulation of mature miRNA levels was expected due to supposedly reduced processing. Only eight miRNAs were significantly up regulated and four miRNAs were significantly down regulated in hypoxia when compared to normoxia.

Some mature and precursor miRNAs were up or down regulated after *DICER* RNA interference in MCF7 cells. Forty five mature miRNAs were up regulated after DICER inhibition when compared to normoxia. This was an unexpected result as mature miRNA levels were expected to be down regulated with DICER inhibition due to the reduction in miRNA processing. It is possible that some of these miRNAs were processed via DICER independent mechanisms. The best reported DICER independent miR-451 (Cifuentes et al., 2010) was not well expressed in MCF7 cells, therefore any changes in miR-451 after DICER inhibition was not observed in this study (Langenberger et al., 2012). However some of the miRNAs identified as DICER independent by Langenberger et al (2012) such as miR-4417, miR-663, miR-4516, miR-3960 and miR-3185 were also up regulated in MCF7 cells after DICER inhibition (Langenberger et al., 2012). In addition, 19 miRNAs were down regulated after DICER inhibition when compared to normoxia, and these miRNAs may be DICER dependent miRNAs. Eight pre-miRNAs were up regulated after DICER inhibition in MCF7 cells, possibly accumulating due to reduced DICER processing.

To determine the influences of hypoxia on processing of each hairpin to a mature miRNA in hypoxia compared to normoxia, the ratios between precursor/mature miRNAs were examined. Even after a longer duration of hypoxic exposure (0.1% O_2 for 48 h) or DICER RNAi, there were no significant changes in precursor/mature miRNA ratios. This observation indicates that there was no accumulation of pre-miRNAs in hypoxia compared to normoxia, due to reduced processing by DICER,

During the course of this work, Ho et al. (2012) reported a reduction in DICER levels after exposure to hypoxia. They reported a decrease in the levels of several mature miRNAs (miR-492, miR-340, miR-184.miR-185, miR-146b-3p, miR-490-3p and

miR-625) and an accumulation of their pre-miRNAs in HUVEC cells after exposure to hypoxia. It was not possible to replicate these findings in this thesis using HUVECs under controlled levels of hypoxia. This suggests that in particular cells or conditions of hypoxic exposure, the hypoxic repression of DICER can have important influences on miRNA biogenesis.

When expression levels of specific miRNAs were examined in MCF7 cells using real time RT-PCR, only a modest decrease in mature levels were observed in hypoxia compared to normoxia. Mature let-7a and miR-21 levels decreased slightly after exposure to hypoxia, and there was no accumulation of pre-let-7a or pre-miR-21 in hypoxia. A significant reduction in mature miR-185 was observed in hypoxia in MCF7 cells, but there was no accumulation of precursors. These miRNAs (let-7a, miR-21 and miR-185) were chosen as previous reports showed they were DICER dependant miRNAs and mature levels decreased with DICER inhibition (Suarez et al., 2007, Ho et al., 2012). Previously Ganiter et al. (2011) explored the surprising stability of many miRNAs and this may largely explain the lack of changes in mature miRNA levels even after significant reduction in miRNA biogenesis proteins (Gantier et al., 2011). They measured decay rates of a few miRNAs (miR-155, miR-21, miR-125b, let-7i, miR-146a, miR-19b, miR-107) in mouse embryonic fibroblasts after deletion of *DICER1*. Using a mathematical model they determined the average half-life of a miRNA to be 119 h (or close to five days) but the half-life varied for each miRNA, from as low as 28 h (miR-155) to 211 h (miR-125b) (Gantier et al., 2011). As shown above in this chapter, the changes in miRNA levels were examined by real time RT-PCR and microarray analysis after exposure to hypoxia for 48 h. It is possible that most miRNAs examined would still have been stable at 48 h and the changes in mature miRNA levels due to disruption of processing would have been

too small to be detected. Suarez et al. (2007) also reported that reducing DICER levels by RNA interference in human endothelial cells did not completely reduce the mature miRNA levels (Suarez et al., 2007). Due to low transfection efficiencies when using RNA interference techniques DICER inhibition could be impaired, therefore miRNA processing might not be affected.

Another possible but unlikely explanation for the lack of altered mature miRNA levels after the down regulation of DICER could be due to the action of DICER independent mechanisms in processing a large number of miRNAs. The operation of DICER independent pathways of miRNA biogenesis have only been well described for a small number of specific miRNAs (Cifuentes et al., 2010, Langenberger et al., 2012). Cifuentes (2010) reported of a DICER independent but AGO2 dependent processing of miR-451 (Cifuentes et al., 2010). Langenberger (2012) reported that a few miRNAs that were processed in a DICER independent manner. Therefore it is unlikely that a large number of miRNAs were processed by a DICER independent mechanism that was unaffected by hypoxia.

Even though only a modest effect of hypoxia on miRNA biogenesis was observed, there was a significant influence of hypoxia on the operation of an exogenously introduced precursor miRNA. The processing and function of miR-200b was reduced in hypoxia when compared to normoxia. DROSHA, DICER and TARBP2 proteins are involved in the processing of a precursor miRNA into a mature miRNA and the reduction of all these proteins in hypoxia would likely have impacted on the maturation of the pre-miR-200b. The reduction in DICER, TARBP2 and AGO2 levels in hypoxia would interfere with the formation and activity of the RISC

complex, and this may further explain the reduced gene silencing in hypoxia when compared to normoxia.

When DICER activity in cell lysates from hypoxic and normoxic cells was measured there were no significant changes in the processing of an introduced pre-miR-145. The processing of pre-miRNA-145 was only slightly reduced in hypoxia when compared to normoxia, even though DICER protein levels were significantly (10-fold) decreased in the hypoxic cell lysates. Therefore another possible explanation could be that the levels of proteins such as DICER are not rate limiting for the production of some miRNAs under these conditions.

6.7 Chapter summary

This chapter examined the functional consequences of the reduction in DICER and other key proteins in hypoxia. The global effects of hypoxia compared to normoxia on mature and pre-miRNA levels were examined using miRNA microarrays and real time RT-PCR techniques. To understand if this reduction in miRNA biogenesis proteins affected the miRNA mediated gene regulation through RISC assembly and function, the effect of hypoxia on the processing and gene suppression of an exogenously introduced precursor miRNA was examined. In addition a DICER activity assay was conducted to examine the influence of hypoxia on the processing of an exogenously introduced pre-miRNA. Even though there was a significant and consistent reduction in the levels of proteins with key roles in miRNA biogenesis under hypoxia, there were no substantial effects on the expression levels of mature miRNAs over the time course of these experiments. While only a modest effect of hypoxia on mature miRNA expression was observed, there was a significant

influence of hypoxia on the operation of an exogenously introduced precursor miRNA. The processing and function of miR-200b was reduced in hypoxia when compared to normoxia. When DICER activity in cell lysates from hypoxic and normoxic cells was examined there were no significant changes in the processing of pre-miR-145, which was only slightly reduced in hypoxia when compared to normoxia. Overall, these observations suggest that early and immediate responses to hypoxic stresses are not likely to be mediated by global alterations in mature miRNA levels.

Chapter 7 Discussion

7.1 DICER regulation by hypoxia

The links between tumour hypoxia, miRNA expression and cancer aggression raised the possibility of a general effect of hypoxia on miRNA biogenesis and function. This thesis investigated the possibility that hypoxic regulation of miRNA biogenesis proteins might contribute to the reduction in miRNA expression in many tumours and the role of hypoxia in cancer progression.

The results from chapter 3 and 5 showed that, several proteins with major roles in miRNA biogenesis (including DICER, TARBP2, DROSHA, DGCR8 and XPO5) were down regulated in hypoxia. DICER mRNA and protein levels were repressed in three different cancer cell lines, two breast cancer cell lines (SKBR3 and MCF7) and one colorectal (HT29) cancer cell line after exposure to hypoxia. In addition hypoxic repression of DICER was also observed in non-cancer HUVECs. These observations suggest that the hypoxic repression of DICER could be a widespread phenomenon in cells and not restricted to cancer cells. Reductions in *DICER* mRNA levels were observed as early as 8 h after hypoxic exposure (0.1% O_2), whereas significant reductions in DICER protein levels were only observed following longer durations (48 and 72 h) and with greater severity of hypoxic exposure (0.1% O_2), possibly due to the stability of DICER proteins. Therefore, the decrease in transcription might not be readily evident until 48 h. Consistent with this explanation, only modest reductions in Dicer protein levels were observed at shorter durations (16 and 24 h) of hypoxic exposure.

Hypoxic regulation of miRNA biogenesis proteins appears to operate at several levels. Whilst *DICER* mRNA down regulation in hypoxia was seen in MCF7 and

HT29 cells, it was not seen in SKBR3 cells even though there was substantial down regulation of DICER protein levels, indicating the operation of transcriptional and post-transcriptional mechanisms in DICER down regulation in hypoxia.

In a previous microarray study, Elvidge et al. (2006) reported a hypoxic down regulation of *DICER* mRNA levels in MCF7 cells. During the course of this work, hypoxic repression of DICER levels was reported by others (Caruso et al., 2010, Ho et al., 2012, Wu et al., 2011). A study using rat pulmonary artery fibroblasts exposed to chronic hypoxia showed reduced *DICER* mRNA expression (Caruso et al., 2010). Also in human pulmonary artery smooth muscle cells (PASMCs) DICER protein levels decreased after exposure to hypoxia (Wu et al., 2011). Ho et al. (2012) demonstrated DICER mRNA and protein down regulation in HUVECs after exposure to hypoxia (1% O_2) for 24 h.

In this thesis a reduction in DICER protein levels was only observed after exposure to hypoxia (1% O_2) for 48 h in HUVECs, and this observation was not in line with the previous observations by Ho et al. (2012). Differeing results may be due to the slight differences in the experimental conditions when exposing cells to hypoxia. In this study HUVECs were exposed to hypoxia using an incubator (see details in chapter 2) where precise O_2 levels could be controlled by balancing with CO_2, N_2 and room air. The use of an incubator with precisely controlled O_2 levels ensured all hypoxic exposures were consistent across different experiments allowing accurate comparisons between multiple experiments. Whereas Ho et al. (2012) used an anaerobic system in which O_2 levels were kept below 1% by flushing with an anaerobic gas mixture which did not allow for precise control of O_2 levels.

To eliminate the possibility of non-specific stress or toxicity effects due to hypoxia, two control proteins (α-actinin and β-tubulin) and two control mRNAs (18S and *β-2-microglobulin*) were examined under hypoxia and normoxia. The expression levels of these controls did not change between hypoxia and normoxia. As shown in chapter 3, a cell viability assay confirmed that, cell numbers between hypoxia and normoxia treatments remained constant. In addition, hypoxically induced proteins such as CAIX and HIF-1 were examined to confirm the induction of such proteins under hypoxic conditions. All experiments were done using a cell culture system under stringently controlled conditions. Therefore these results might not be directly comparable to an *in vivo* model or to an actual tumour condition.

7.2 Mechanisms of DICER regulation in hypoxia

In exploring the mechanism of hypoxic regulation of DICER, the role of HIF and the HIF hydroxylases was examined. When cells were exposed to the HIF hydroxylase inhibitors DMOG and desferroxiamine a modest decrease in *DICER* mRNA and protein levels was observed in MCF7 cells. However, somewhat surprisingly no, or very modest, effects on the repression of DICER mRNA and protein levels was observed after HIF-1α and HIF-2α inhibition with RNA interference. No change in DICER levels was observed after HIF-1α and HIF-2α over expression in normoxia. These observations suggested that despite sensitivity to HIF hydroxylase inhibitors, HIF did not play a major role in the hypoxic down regulation of DICER, a conclusion also reached recently by Ho and colleagues. Ho et al. (2012) reported in HUVECs that, DICER repression in hypoxia was not dependent on HIF-2α which is the predominant HIF-α isoform in endothelial cells (Ho et al., 2012). In addition they also looked at the DICER promoter region (1500 bp of the transcription start site) of

the most commonly expressed *DICER* mRNA variant and did not find hypoxia response elements (Ho et al., 2012).

The VHL protein regulates the degradation of HIF-α subunits in normoxia by binding to the hydroxylated proline residues of the HIF-α subunits, and facilitating ubuquitination followed by proteasomal degradation (Maxwell et al., 1999). In chapter 4 of this thesis VHL dependent regulation of DICER mRNA and protein levels was examined using RCC4 cells with and without the VHL function. DICER down regulation was not observed in both cell types, in hypoxia or in normoxia. HIF-1 levels were increased in RCC4 -VHL cells when compared to RCC4 +VHL cells even in normoxia, but there was no effect on DICER levels between these cell lines. In contrast Ho et al. (2012) reported that DICER protein levels were lower in VHL deficient RCC4 and UMRC2 or VHL mutant 786-O-CI62F cells when compared to the VHL expressing cells suggesting at least some VHL mediated DICER regulation (Ho et al., 2012). Furthermore they showed that in hypoxia, DICER mRNA and protein levels decreased in the VHL wild-type cells but not in VHL deficient RCC4 cells (Ho et al., 2012). The lack of DICER regulation observed in these two cell lines (RCC4 –VHL and RCC4 +VHL) further points towards HIF independent regulation. However, it is important to note that there was no difference in DICER expression even in the wild type RCC4+VHL cells when exposed to hypoxia or normoxia.

Taken together, these observations suggested a mechanism of DICER regulation that was mediated by oxygen and HIF hydroxylases but was independent of HIF. To explore this mechanism further, the possible contribution of each of the three HIF prolyl hydroxylases (PHD1, 2 and 3) and the HIF asparaginyl hydroxylase, FIH-1 on DICER regulation was examined. When *PHD2* was suppressed by RNA interference

in normoxic conditions, significant decreases in DICER mRNA and protein levels was observed. This indicates the operation of another pathway of response to hypoxia in which PHD2 is playing a central role. A previous study showed that PHD2 regulates EEF2 phosphorylation thus, affecting protein translation (Romero-Ruiz et al., 2012) and this might partly explain the global protein down regulation observed in hypoxia. PHD2 is also involved in down regulating NF-κB, another transcription factor that operates in a HIF independent manner in hypoxia (Chan et al., 2009). Therefore, PHD2 dependent DICER regulation could be HIF independent but through NF-κB activation. Further investigation is needed to identify other transcription factors that might be involved in the regulation of miRNA biogenesis machinery in hypoxia independently of HIF. The other HIF hydroxylase enzymes (PHD1, PHD3) and FIH-1 did not appear to be involved in DICER regulation in hypoxia.

The DICER 3'UTR contains conserved binding sites for the miR-103/107 family (Martello et al., 2010) and let-7a (Forman et al., 2008). Given the importance of miRNA mediated feedback loops in the control of DICER expression (Forman et al., 2008, Martello et al., 2010), chapter 4 of this thesis examined the possible influence of hypoxically induced miRNAs in mediating the hypoxic repression of DICER. Hypoxically induced miR-210 is the best characterised example of a miRNA that shows substantial induction under hypoxic conditions (Kulshreshtha et al., 2007a, Kulshreshtha et al., 2007b). However, the introduction of miR-210, or its antagonism, had only a small or no effect on DICER protein levels, arguing against a major role in the hypoxic regulation of DICER. The TargetScan a miRNA target prediction tool reported of a large number of miRNA target sites on the 3'UTR of DICER (TargetScan release 6.2) (Friedman et al., 2009), therefore its possible that

manipulating the levels of a single miRNA might not have a significant effect on DICER expression.

The two miRNAs, miR-103 and miR-107 have been shown to decrease miRNA biogenesis by targeting DICER in cancer (Martello et al., 2010) and have been reported to show hypoxic induction in some situations (Kulshreshtha et al., 2007b). A feedback loop exists between these two miRNAs and DICER, in order to maintain DICER expression at low levels, but enough to sustain tumour growth (Martello et al., 2010). The levels of miR-103 and miR-107 showed a modest increase in MCF7 cells after exposure to hypoxia. To examine their contribution to hypoxic repression of DICER, cells were exposed to hypoxia after inhibiting miR-103 and miR-107 with antagomirs. The consistent and significant down regulation of DICER levels in hypoxia was reversed after inhibiting miR-103 and miR-107. These results demonstrate the involvement of miR-103/miR-107 feedback loops in the hypoxic regulation of DICER and were consistent with a role for miR-103 and miR-107 in the regulation of DICER (Martello et al., 2010).

As the DICER 3'UTR contains conserved binding sites for some miRNAs (Martello et al., 2010, Forman et al., 2008) the possibility of miRNA mediated regulation of DICER 3'UTR in hypoxia was examined. Luciferase activity was measured in cells transfected with a luciferase construct containing the firefly luciferase coding sequence fused to the full length DICER 3"UTR (Lux-DICER-3'UTR) and exposed to hypoxia or normoxia (Martello et al., 2010). There was no hypoxic repression of this construct and in fact there was a significant enhancement of luciferase activity under hypoxic conditions, arguing against a role for miRNAs targeting the 3'UTR of DICER in regulation of its hypoxic repression. However, it is important to note some

limitations of reporter assays. The reporter expression may be altered based on the region chosen for cloning and can be sensitive to changes in protocol such as the method of transfection or promoter identity (Kong et al., 2008, Thomson et al., 2011).

Proteins in mammalian cells are mainly degraded by proteasomes. The involvement of proteasomes in DICER protein repression during hypoxia was examined using a proteasomal inhibitor (MG-132). There was no change in the hypoxic repression of DICER protein levels after treatment with the proteasomal inhibitor, suggesting that proteasomes are not involved in DICER regulation in hypoxia. However, the control (DMSO only) treatment in hypoxia did not show a decrease in DICER protein levels, making it difficult to interpret these results. A previous study by Ho et al. (2012) showed that proteasomes did play a role in DICER regulation. They saw DICER repression being reversed in hypoxia after treatment with a proteasomal inhibitor (MG-132) in HUVECs (Ho et al., 2012).

7.3 Hypoxia down regulates other miRNA biogenesis proteins

Other proteins involved in miRNA biogenesis also showed significant down regulation under hypoxia when compared to normoxia. Consistent down regulation of DROSHA, DGCR8, TARBP2, XPO5 and AGO2 protein levels were observed after exposure to hypoxia (0.1% O_2 for 48 h). A previous microarray study also showed modest but consistent hypoxic reductions in mRNA levels of genes (DROSHA, TARBP2 and AGO2) involved in miRNA biogenesis in MCF7 cells (Elvidge et al., 2006). Ho et al. (2012) have also reported a decrease in DGCR8, XPO5 and AGO2 protein levels in HUVECs after exposure to hypoxia, (though they

did not see a decrease in DROSHA levels in hypoxia) (Ho et al., 2012). Wu et al. (2011) also reported a down regulation of DICER and DROSHA protein levels in human primary pulmonary artery smooth muscle cells (PASMCs) after exposure to hypoxia. In addition, they reported an increase in AGO2 levels in PASMCs after exposure to hypoxia due to the type 1 collagen prolyl-4-hydroxylase (C-P4H(1)) dependent hydroxylation and stabilisation of AGO2 (Wu et al., 2011). These observations by Wu et al. (2011) contradict the results in this thesis and Ho et al. (2012), where a decrease in AGO2 levels were observed in MCF7 cells and HUVECs after exposure to hypoxia.

These observations suggest the operation of a broader influence of hypoxia on the expression of proteins that are essential in miRNA biogenesis. Exposure to stress conditions such as hypoxia can lead to down regulation of protein synthesis as a mechanism of energy conservation in the cell (Koritzinsky et al., 2005, Wouters et al., 2005). The global down regulation of protein synthesis in response to cellular stress, could be partly responsible for the down regulation of several proteins involved in the miRNA biogenesis pathway in hypoxia.

Another possible explanation could be that these proteins regulate each other by forming complexes and the decrease or destabilisation of one protein might have an effect on the stability of another protein. Previously others have shown a powerful influence of the levels of DICER protein on the levels of TARBP2 and vice versa (Chendrimada et al., 2005, Melo et al., 2009) and the post transcriptional cross regulation between DROSHA and DGCR8 (Han et al., 2009). When DICER expression was suppressed using RNA interference, a significant decrease in TARBP2 levels was observed in keeping with previous reports (Melo et al., 2009).

Similarly, when TARBP2 levels were reduced by siRNA treatment there was a modest decrease in DICER protein levels. When DICER and TARBP2 levels were examined over a time course of hypoxic exposure, both proteins seemed to decrease co-ordinately. Similarly, Melo et al. (2009) observed that TARBP impairment is associated with a destabilization of DICER and the loss of TARBP2 leads to a defect in DICER activity. In contrast a study by Hasse et al. (2005) did not see destabilisation of TARBP2 when DICER was depleted or vice versa (Hasse et al. 2005).

As illustrated in chapter 5 both DROSHA and DGCR8 levels significantly decrease in hypoxia. Previously Han et al. (2009) showed that DROSHA and DGCR8 regulate each other post transcriptionally (Han et al., 2009). DROSHA down regulates *DGCR8* mRNA stabilisation, therefore the inhibition of DROSHA by RNA interference resulted in an increase in DGCR8 protein levels. DGCR8 stabilised DROSHA protein levels via a protein-protein interaction, and inhibition of DGCR8 by RNA interference decreased DROSHA protein levels (Han et al., 2009). It is possible that a similar mechanism operates between DROSHA and DGCR8 in hypoxia and the effect of hypoxia on DGCR8 might be affecting DROSHA protein stabilisation and vice versa.

Chapter 5 also showed a significant down regulation of XPO5 in hypoxia. A similar observation was reported by Ho et al. (2012) in HUVECs after exposure to hypoxia. Previous work showed that XPO5 is involved in DICER regulation post transcriptionally by decreasing the DICER mRNA export from the nucleus (Bennasser et al., 2011). Otherwise XPO5 is not involved in mRNA transport and only known to transport tRNAs, Y-RNAs and pre-miRNAs (Bennasser et al., 2011).

It is possible that the decrease in XPO5 due to hypoxia reduced DICER mRNA transport from the nucleus therefore down regulating DICER protein levels in hypoxia.

A further relationship linking DICER and DROSHA expression was observed. There was an increase in DROSHA protein levels after inhibiting DICER, and this has previously been observed in HeLa cells (Bennasser et al., 2011). A decrease in DICER protein levels was observed after DROSHA suppression by RNA interference. Further investigation is required to understand the mechanisms involved in this regulation, and miRNA mediated effects are highly plausible. Recent work has shown that AGO2 stability is dependent on the availability of mature miRNAs. DICER knockout influenced the mature miRNA production leading to decreased AGO2 stability (Smibert et al., 2013).

As discussed above, RNA interference was used for inhibiting specific genes and all of the siRNAs used in this study have been previously validated by other groups. However, unintended gene modulation by off-target effects due to different mechanisms is a concern when using RNA interference for gene inhibition, and this can complicate the interpretation of phenotypic effects and also lead to unexpected toxicities (Jackson and Linsley, 2010).

7.4 Effect of hypoxia on mature miRNA expression and function

Despite a significant and consistent reduction in the levels of proteins with central roles in miRNA biogenesis machinery under hypoxia, a substantial effect on mature miRNA expression was not observed over the time course of these experiments.

DICER suppression by RNA interference was only associated with slight alterations in mature miRNA abundance. This was true both for miRNAs assessed by microarray studies and also when examining the levels of particular miRNAs and their precursors by real time RT PCR. In this study microarray data was normalised using the quantile normalization method, in which high intensity data was changed into the same distribution shape as the rest of the data. This artificial change might reduce the biological significance as well as technical differences, therefore making it difficult to detect subtle biological changes.

In MCF7 cells only a modest decrease in mature and precursor levels of let-7a and miR-21 was seen after exposure to hypoxia, and an accumulation of pre-let-7a or pre-miR-21 in hypoxia was not observed. A significant reduction in mature miR-185 was observed in hypoxia in MCF7 cells, but there was no accumulation of precursors. These miRNAs (let-7a, miR-21 and miR-185) were chosen as previous reports showed they were DICER dependant miRNAs and mature levels decreased with DICER inhibition (Suarez et al., 2007, Ho et al., 2012, Bu et al., 2009). The use of knockout murine models has provided compelling evidence for the essential role of DICER in mature miRNA production. However, it has proven more difficult to clearly see such critical influences in a variety of experiments that have utilised siRNA mediated DICER suppression (Suarez et al., 2007, Yang and Lai, 2011). In a previous study Yi et al. (2003) reported the importance of XPO5 binding for pre-miRNA stability in the nucleus. They observed a decrease in pre-miRNA levels after depletion of XPO5 (Yi et al., 2003). The lack of pre-miRNA accumulation in hypoxia described in chapter 6 could be due to the reduced stability of pre-miRNAs in hypoxia, as a result of reduced XPO5 expression in hypoxia.

Others have previously explored the surprising stability of many miRNAs and this may largely explain the lack of changes in mature miRNA levels even after significant reduction in miRNA biogenesis proteins (Gantier et al., 2011). They measured decay rates of a few miRNAs (miR-155, miR-21, miR-125b, let-7i, miR-146a, miR-19b, miR-107) in mouse embryonic fibroblasts after the deletion of DICER. Using a mathematical model they determined the average half-life of a miRNA to be 119 h (or close to five days) but the half-life varied for each miRNA from as low as 28 h (miR-155) to 211 h (miR-125b) (Gantier et al., 2011). As shown in chapter 6 the change in miRNA levels after exposure to hypoxia was examined after 48 h. It is possible that most miRNAs examined would have been stable at 48 h and the changes in mature miRNA levels due to disruption of processing would have been too small to be detected.

The operation of DICER independent pathways in the generation of miRNAs has been well described for a small number of specific miRNAs (Langenberger et al., 2012, Cifuentes et al., 2010, Cheloufi et al., 2010). This could be a possible explanation for the lack of influence of DICER down regulation in hypoxia on global miRNA abundance. However, considering the very small number of DICER dependent miRNAs described so far, it is an unlikely explanation. The best reported DICER independent miRNA miR-451 (Cifuentes et al., 2010) was not well expressed in MCF7 cells therefore any changes in miR-451 after DICER inhibition could not be observed in this study. Some of the miRNAs identified as DICER independent by Langenberger et al. (2012) such as; miR-4417, miR-663, miR-4516, miR-3960 and miR-3185 were also up regulated in MCF7 cells after DICER inhibition (see chapter 6).

There is also the possibility that the levels of proteins such as DICER are not rate limiting for the production of miRNAs under these conditions. These observations also imply that early and immediate responses to hypoxic stresses such as the down regulation of several key proteins in miRNA biogenesis are not likely to result in global alterations in mature miRNA levels.

During the course of this work, Ho and colleagues also described a reduction in DICER levels after exposure to hypoxia, and a decrease in the levels of several mature miRNAs (miR-492, miR-340, miR-184, miR-185, miR-146b-3p, miR-490-3p and miR-625) and an accumulation of their pre-miRNAs in HUVEC cells (Ho et al., 2012). It was not possible to replicate these findings using controlled levels of hypoxia. As discussed above this may suggest that in particular cells or conditions of hypoxic exposure, the hypoxic repression of DICER can have important influences on miRNA biogenesis.

Even though hypoxia exerted only modest effects on the production of mature miRNAs, a significant influence of hypoxia was observed on the operation of an exogenously introduced precursor miRNA. The processing and function of miR-200b was reduced in hypoxia when compared to normoxia. DROSHA, DICER and TARBP2 proteins are involved in the processing of an exogenously transfected precursor miRNA containing flanking nucleotides into a mature miRNA and the reduction of both these proteins in hypoxia would likely have impacted on the maturation of the pre-miR-200b.

When DICER activity from hypoxic and normoxic cell lysates was examined, there were no significant changes in processing a pre-miR-145. The processing of the pre-

miRNA-145 was only slightly reduced in hypoxia when compared to normoxia, even though DICER protein levels were significantly (10 fold) decreased in hypoxia. This reconfirmed the previous observation that changes in DICER protein availability did not significantly affect mature miRNA levels in cancer cells.

7.5 Significance

Research findings from this study provide evidence for an important potential interface between oxygen availability and gene expression via alterations in the levels of proteins critical for miRNA generation. This study provides evidence for the existence of a co-ordinated mechanism of repression of miRNA biogenesis proteins in response to hypoxia. In addition, it demonstrates a potential mechanistic explanation for the reduced levels of miRNAs observed in some cancers when compared to normal tissue. This study identified two mechanisms that are involved in the hypoxic regulation of miRNA biogenesis proteins. The first was a PHD2 dependent but HIF independent mechanism of DICER down regulation in hypoxia. The second mechanism was a specific miRNA (miR-103/107) mediated feedback loop in the regulation of DICER proteins under hypoxic conditions. These findings present examples of mechanisms for protein repression in hypoxia and also provide further support for the existence of feedback mechanisms in the regulation of the miRNA biogenesis pathway. Some of these miRNA biogenesis proteins are involved in other functions, in addition to miRNA biogenesis, and the hypoxic repression of these proteins may impair those functions (Wu et al., 2000, Meister, 2011). For example DICER processes longer *Alu* RNAs into shorter non-toxic sequences in retinal pigment epithelium (Meister, 2011). DROSHA has previously been shown to also function in ribosomal RNA processing in addition to miRNA processing (Wu et al., 2000).

7.6 Future directions

Whilst this work has provided evidence for the mechanism and regulation of DICER and other miRNA biogenesis proteins by hypoxia, it has also highlighted the following questions:

1. Under what physiological and pathological circumstances does the hypoxic reduction of miRNA biogenesis proteins operate?
2. What mechanisms and relationships exist between miRNA biogenesis proteins and hypoxia in cancers?
3. What is the effect of hypoxia on miRNA based therapies?

Further investigation is needed to understand the functionally important effects of miRNA abundance in hypoxia. Relevant physiological and pathological circumstances under which hypoxic reduction of miRNA biogenesis proteins operates also need to be examined. This would include investigating the links between hypoxia, miRNA expression and miRNA biogenesis protein levels in solid tumours and in cells after prolonged exposure to hypoxia.

Examining the relationship between DICER (or other miRNA biogenesis proteins), HIF-1 and CAIX protein levels in a cohort of solid tumours using standard immunohistochemical staining methods would clarify the relationship between hypoxic tumours and miRNA biogenesis protein levels in cancers.

Results from this study did not show a significant effect of hypoxia on mature miRNA levels after 48 h, possibly due to the stability of existing miRNAs. In this study, cells were only exposed to 48 h of hypoxia (0.1% O_2), because longer term

exposure of MCF7 cells or SKBR3 cells to 0.1% O_2 detrimentally affected cell proliferation, thereby significantly changing the number of cells between treatments. Some cell lines (e.g. HCT116) have been reported to survive and grow in long term hypoxia (Yu and Hales, 2011). It would be interesting to investigate the effect of exposure to longer periods of hypoxia on miRNA biogenesis proteins and mature miRNA expression using different cell lines.

In order to understand the relevance of the findings from this study in an *in vivo* model, miRNA biogenesis protein levels and mature miRNA production in hypoxia could be examined in xenograft and orthotopic mouse models. Ho et al. (2012) observed decreases in DICER protein levels in several organs after exposing mice to 8% O_2 for 24 h, and also observed a down regulation of several miRNAs in the mouse kidney in hypoxia. To understand the effect of hypoxia in an *in vivo* tumour model Yu et al. (2011) examined rats bearing transplanted tumours and observed that while lung cancer tumour growth significantly reduced in long term hypoxia, it had the opposite effect on colon cancer tumour growth which was significantly increased in long term hypoxia. Examining DICER expression and mature miRNA expression in these transplanted tumours under hypoxia would determine if miRNA biogenesis proteins decreased in hypoxia, and if the increase or decrease in tumour growth correlated with mature miRNA levels in hypoxia.

Recent reports demonstrated the important potential of using miRNA based therapies for cancer (Kota et al., 2009, Esquela-Kerscher et al., 2008, Kumar et al., 2008). Kota et al. (2009) demonstrated that systemic administration of adeno-associated virus (AAV) mediated delivery of miRNAs suppressed tumour growth in murine liver tumours (Kota et al., 2009). It would be interesting to investigate whether the

functions of such miRNA based therapies are impaired due to the lack of miRNA biogenesis proteins in hypoxic tissues.

This study reported a PHD2 mediated repression of DICER mRNA and protein levels. As mentioned earlier, a recent study reported a PHD2 dependent down regulation of proteins in acute hypoxia via the PHD2 mediated phosphorylation of EEF2 and the disruption of protein translation (Romero-Ruiz et al., 2012). Further investigation is needed to understand the detailed mechanisms involved in this PHD2 mediated down regulation of DICER. To answer the question as to whether EEF2 phophorylation is directly affecting DICER down regulation in hypoxia, DICER levels could be examined in cells after over expressing EEF2 in hypoxia.

7.7 Conclusion

Hypoxia exerts substantial effects on the expression of DICER and several other proteins required for the synthesis of mature miRNAs. This effect of hypoxia appears to operate via several mechanisms of regulation and this thesis has shown important influences of the HIF hydroxylase PHD2 and the hypoxically regulated miRNAs miR-103 and miR-107 in this regulation. Furthermore, co-ordinated regulation of several proteins involved in miRNA biogenesis is apparent under hypoxic conditions. The influences on mature miRNA production were more modest than might have been anticipated from the substantial hypoxic reductions in protein levels of DICER, DROSHA, TARBP2 and XPO5. The breadth of this effect suggests a further and important interface between oxygen availability and gene expression.

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